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THE BRITISH JOURNAL OF NUTRITION

THE BRITISH JOURNAL OF NUTRITION

INCORPORATING THE PROCEEDINGS OF THE NUTRITION SOCIETY

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VOLUME 6, 1952

CAMBRIDGE
AT THE UNIVERSITY PRESS

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press

London: Bentley House, N.W.1
American Branch: New York

Agents for Canada, India, and Pakistan: Macmillan

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The Nutrition of the Young Ayrshire Calf

5. The Nutritive Value of Cow's Whole Milk

BY K. L. BLAXTER (IN RECEIPT OF A SENIOR AWARD OF THE AGRICULTURAL RESEARCH COUNCIL)
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(*Received* 1 *March* 1951)

The energy and nitrogen metabolism of calves, when given whole-milk diets, has been the subject of experiment for over 70 years. Soxhlet's (1878) work involved simultaneous study of mineral, N and energy balances and these experiments, together with those of Fingerling (1908), Blackwood, Morris & Wright (1936) and of Tomme & Taranenko (1939), appear to be the only ones that have not been complicated by the inclusion of roughage in the diet of the animals. No metabolism studies appear to have been made in which variation in nutritional plane has been employed. Several practical trials have, however, been carried out in which different quantities of whole milk have been given (Filmer, 1948, 1949) and in which higher planes of nutrition have been associated with greater growth, at least during the first 5 weeks of life.

In a previous paper (Blaxter & Wood, 1951*c*) it was suggested that biological values of proteins, giving their true significance, could be determined by regression analysis of the results of nitrogen-balance experiments in which different quantities of the same diet had been given. The object of the present experiment was to repeat this type of determination using cow's whole milk. In view of the paucity of information on the calcium and phosphorus metabolism and the energy exchange of the young calf in relation to nutritional plane, additional observations on these aspects were made.

EXPERIMENTAL

Calves and their management

Two bull calves, which had previously been given colostrum and whole milk, were used. They were housed in metabolism crates; urine and faeces collections were made as previously described (Blaxter & Wood, 1951*a*). The calves were 5 days old at the commencement of the experiment. Whole milk was given to each calf as its sole diet for three 12-day periods; calf no. 13 received 2·6, 5·0 and 5·8 l. in consecutive periods and calf no. 14 received 4·2, 3·4 and 6·6 l. These quantities are step-wise changes of 0·8 l. and were chosen to cover a range of feeding from about maintenance level to two and a half times maintenance. The diets were allocated in such a way that each calf was given widely differing quantities. Two days were allowed between each change-over to allow an equilibrium to be attained. The whole milk used contained on the average 3·0–3·1 % fat and was supplemented by a daily dose of 5 ml. of a mineral mixture containing those trace elements that are required by the rat when milk is the sole diet. Magnesium was also included in this mixture to prevent the hypomagnesaemic tetany of calves that results when whole-milk diets are given (Duncan, Huffman & Robinson, 1935). The composition of this mixture in g/l. was: $MgSO_4.4H_2O$ 0·5, $CuSO_4.5H_2O$ 0·5, $CoSO_4.7H_2O$ 0·1, $Fe_2(SO_4)_3.7H_2O$ 20·0, $MgCl_2.6H_2O$ 60·0. Five ml. cod-liver oil were given once a week.

Milk samples were taken at each feeding (9 a.m. and 8 p.m.) and were preserved with formaldehyde. Urine and faeces were collected daily.

Analytical procedure

All analyses for nitrogenous constituents were made at 2-day intervals with the exception of urinary N which was determined daily. Mineral analyses were made on pooled samples representing the last 10 days of each period. The animals were weighed every 2 days before the morning feed. Notes on general behaviour and appearance were made at each feeding and the pulse rate was also recorded at this time. The respiratory metabolism was determined using the apparatus and technique of Blaxter & Howells (1951).

The following determinations were made:

Urine. Total N (Kjeldahl); urea (Van Slyke & Cullen, 1910); ammonia (Van Slyke & Cullen, 1910); protein (Kjeldahl); amino-N (Albanese & Irby, 1944); purine bases (Krüger & Schmidt, 1905); uric acid (King, 1946); allantoin (Young & Conway, 1942); creatine and creatinine (Folin, 1914); calcium (McCrudden, 1911–12); phosphorus (Fiske & Subbarow, 1925).

Faeces. Dry matter; total N (Kjeldahl); ash; total fat.

Milk. Fat (Röse-Gottlieb); total N (Kjeldahl); total solids; ash; calcium (McCrudden, 1911–12); phosphorus (Fiske & Subbarow, 1925).

RESULTS

General observations and body-weight gains

Both calves were bright and in good condition at the commencement of the experiment. Calf no. 13, when receiving the lowest quantity of milk (2·6 l./day) became rather thin, but still remained alert and active. Towards the end of period 3 this calf became lethargic and scoured badly. Calf no. 14 was in excellent condition throughout the experiment. Calf no. 13 was more nervous and irritable than calf no. 14.

Table 1 summarizes the daily body-weight gains calculated by regression analysis of the individual weights, and these values plotted against milk intake are presented in Fig. 1. It will be noted that the relation between the rate of gain of body-weight and milk intake was linear, even at the highest level of feeding. Though the results are limited in that they apply to only two calves, they have been analysed statistically using methods of covariance analysis (Fisher, 1925). The method is shown in the second part of Table 1 and has been used throughout. It makes possible an estimate of the

Table 1. *Gain in body-weight of the calves and its statistical significance*

Calf no. 13		Calf no. 14	
Milk ingested (l./day)	Body-weight gain (g/day)	Milk ingested (l./day)	Body-weight gain (g/day)
2·6	−49	3·4	230
5·0	480	4·2	355
5·8	690	6·6	910

Analysis of covariance of body-weight gain in g/day (y) on milk intake in l./day (x)

	Degrees of freedom	Sum of squares and products			Reduction in sum of squares due to regression	Sum of squares after reduction	Mean square
		x^2	xy	y^2			
Between calves	1	0·107	49·87	23,313	—	6333	6333
Within calves	4	11·093	2470·13	552,037	550,020	2017	672
Total	5	11·200	2520·00	—	567,000	8350	—

Significance of regression: variance ratio (3 degrees of freedom) = 818·2, $P < 0·001$.
Significance of differences between calves: variance ratio (3 degrees of freedom) = 9·4, $P = 0·054$.

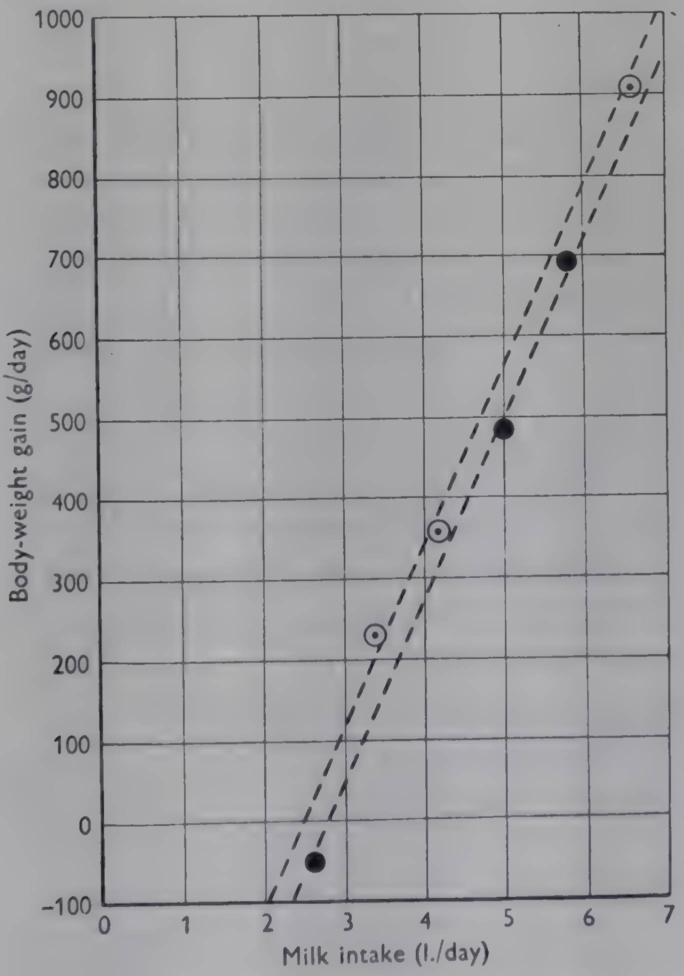

Fig. 1. Relation between milk intake and gain in body-weight of the calves. ⊙, calf no. 14; ●, calf no. 13.

significance of the regression of body-weight gain, N balance or other balances on milk intake as well as an assessment of differences between the two calves when given identical quantities of milk. To establish a difference in performance of two animals in such a short period as the first 6 weeks of life is difficult, since the length of period precludes replication on anything but a limited scale. Even so, statistical analysis of such limited data provides a reasonably valid estimate of the significance of any differences. The two equations relating body gain in g/day (G) to milk intake in l./day (M) were:

$$\text{calf no. 13} \quad G=222{\cdot}7M-621{\cdot}8,$$
$$\text{calf no. 14} \quad G=222{\cdot}7M-555{\cdot}7.$$

As shown in the analysis of variance, the pooled regression was highly significant statistically and the difference between calves, after allowing for differences in intake of milk, was only significant at $P=0{\cdot}054$. From this it would appear that calf no. 14 was a more efficient animal than calf no. 13. These two equations permit the estimation of the milk required for maintenance of body-weight, that is the milk intake associated with no gain of body-weight. For calf no. 13 this was 2·8 l./day, and for calf no. 14, 2·5 l., with a mean of 2·65 ± 0·16, which may be taken as an estimate of the requirement of the calf for body-weight maintenance in terms of milk with a fat content of 3 %.

The apparent digestibility of the milk

From analyses of faeces the coefficients of apparent digestibility of dry matter, N and fat were calculated as shown in Table 2. There were no statistically significant differences in the digestibility of any component due to either the effect of the amount of milk consumed, or to individual differences between the calves. Low apparent digestibility of dry matter, of N and, more particularly, of fat occurred when calf no. 13 scoured during period 3. The mean apparent digestibility of N, irrespective of calf or feeding level, was 93·8 %. This value is quite comparable to that of 93·3 % obtained by Blackwood *et al.* (1936), that of 94·3 % obtained by Tomme & Taranenko (1939) and that of 95·4 % obtained by Fingerling (1908). The mean apparent digestibility of the milk fat was 95·6 %. Fingerling obtained a value of 100 for the apparent digestibility coefficient of the ether extract of milk. This high value was no doubt due to the fact that his fat determinations were made on dried, unacidified faeces, so that soaps,

Table 2. *Apparent digestibility for the calves of dry matter, nitrogen and fat*

		Mean daily faecal excretion (g)			Coefficient of apparent digestibility (%)		
Calf no.	Milk ingested (l./day)	Dry matter	N	Fat	Dry matter	N	Fat
13	2·6	12·8	0·88	5·0	96·3	94·1	93·4
14	3·4	19·6	1·83	4·0	95·4	90·7	96·1
14	4·2	15·2	1·50	4·4	97·0	93·8	96·4
13	5·0	11·3	0·85	3·2	98·2	97·1	97·9
13	5·8	38·8	2·70	13·0	94·4	91·7	92·8
14	6·6	19·5	1·64	6·5	97·5	95·6	96·8

normally present in large quantities in calf faeces, would not be included in the total fat excretion. Tomme & Taranenko (1939) give a value of 95 % for the 'digestibility' of the total calories of cow's milk. The present results are thus in substantial agreement with previous results. Except for calf no. 13, during the period when it had diarrhoea, the faecal excretion of fat remained very constant despite the wide range of dietary intake (from 75 to 205 g fat daily). This suggests that most of the faecal fat is of body origin and does not represent unabsorbed dietary fat. Even during fasting, the calf excretes 4·2 g fat/day (Blaxter & Wood, 1951*b*), a value close to the mean excretion of 4·6 g observed in these experiments when the one abnormal value was excluded.

Nitrogen metabolism

Table 3 summarizes the results from which the N balances may be calculated.

When the N balances were plotted against the dietary N apparently digested as shown in Fig. 2, it was seen that the calves differed in their N balances in much the same way as they did in their body-weight gains, calf no. 14 storing more N than calf no. 13 at comparable intakes. Employing the statistical method used in the analysis of weight gains, it was found that the difference in intercept was statistically significant ($P<0{\cdot}01$), so that there were two equations relating N balance (NB) in g/day to apparently digested N intake in g/day (ADN):

Table 3. *Nitrogen metabolism of the calves in relation to milk intake*

Calf no.	Milk ingested (l./day)	N intake (g/day)	N apparently digested (g/day)	Urinary N (g/day)	N balance (g/day)
13	2·6	14·96	14·08	13·54	0·54
14	3·4	19·78	17·95	9·53	8·41
14	4·2	24·16	22·66	8·74	13·92
13	5·0	29·09	28·24	12·58	15·66
13	5·8	32·36	29·66	13·63	16·03
14	6·6	37·03	35·39	10·31	25·07

$$\begin{aligned} &\text{calf no. 13} \quad NB=0{\cdot}972ADN-12{\cdot}8,\\ &\text{calf no. 14} \quad NB=0{\cdot}972ADN-\ 9{\cdot}2,\\ &\text{mean} \quad NB=0{\cdot}972ADN-11{\cdot}0. \end{aligned}$$

The intercepts of the equations represent the loss of body N when the N intake is nil, and, as apparently digested nitrogen is the independent variable, they also represent the urinary N excretion under the same conditions. The mean value of the intercept, 11·0 g, is higher than was found with diets containing various levels of dried skim-milk protein where these intercepts increased with increasing level of dietary protein (Blaxter & Wood, 1951*c*). Blaxter & Wood suggested that this intercept represents two fractions, one endogenous and one largely determined by the higher levels of deamination which occur on high-protein diets. The present result is in complete accordance with those results, for in cow's milk 29·7 % of the total calories are present as protein, whereas in the previous experiments the maximum was 20·5 %. Statistically, the regression of urinary N excretion on N intake was not significant. This is a reflexion of the high biological value of whole-milk protein, for the additional amounts of protein given were all stored. The difference between the calves in their urinary N excretion, however, was statistically significant ($P<0.01$). The implications of this difference will be discussed later.

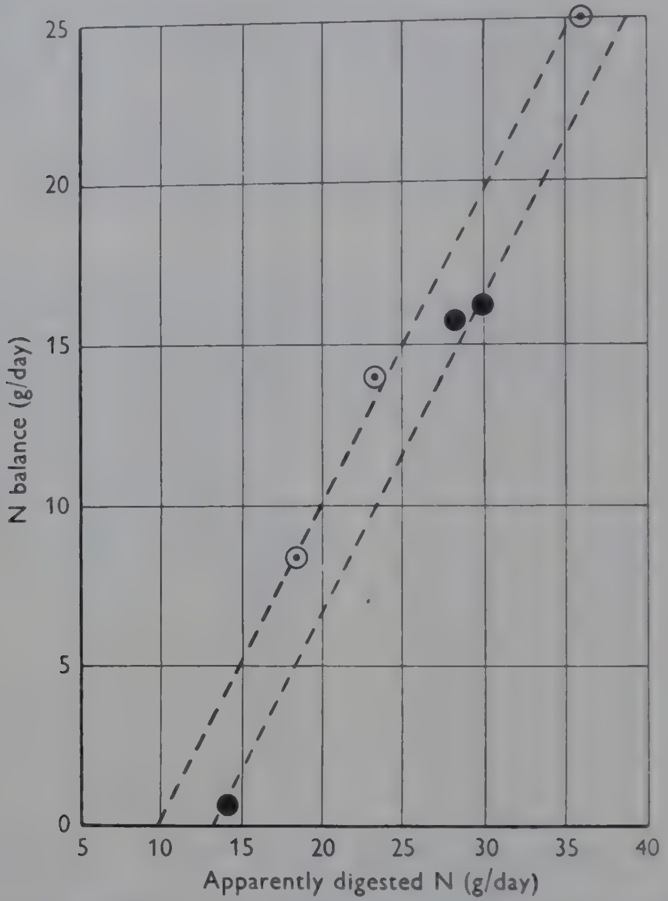

Fig. 2. Relation between N balance of the calves and N apparently digested. ⊙, calf no. 14; ●, calf no. 13.

In each period the distribution of the urinary N was determined. As may be inferred from the results for total urinary N excretion, only small changes occurred as the result of a change in nutritional plane. Creatinine N increased ($P=0.07$) with increasing intake of milk. There were, however, large and statistically significant differences between the calves in their excretion of urea, ammonia and amino-acid N, as shown in Table 4. An increase in the excretion of these three metabolites accounted for over 90 % of the difference between the total N excretion of the calves. The small difference between the calves in their excretion of purine N and creatinine N was not statistically significant. It may be concluded therefore that the higher N excretion by calf no. 13 was the result of an increased protein metabolism, rather than of an increase in its endogenous metabolism, as indicated by purine and creatinine metabolism.

It will be noted that a heat-coagulable protein is a normal minor constituent of calf urine. Smith & Little (1924) reported pronounced proteinuria in newborn calves after they had been given colostrum, but found none after 3 days of age, regarding the fine clouding then present after the heat test as negative.

Biological values of the dietary N were calculated as in previous papers, using as an estimate of the endogenous N excretion a value calculated from basal-energy metabolism determinations made simultaneously (Blaxter & Wood, 1951*a*). These values are presented in Table 5. The biological value of the whole-milk N increased with increasing intake in both calves. The regression of biological value on milk intake was statistically significant ($P=0.03$), and this result confirms previous work on semi-synthetic diets which showed that the biological value of a protein in a growing animal varies with the total intake of food, provided the composition of the diet remains constant (Blaxter & Wood, 1951*c*).

Table 4. *Mean daily excretion by the calves of nitrogen in urinary nitrogenous metabolites (g)*

Metabolite	Calf no. 13	Calf no. 14
Urea	8·92	6·22
Ammonia	1·18	0·73
Amino-acids	0·34	0·23
Total urea, ammonia and amino-N	10·44	7·18
Purine bases	0·16	0·14
Uric acid	0·04	0·04
Allantoin	0·59	0·55
Total purine	0·79	0·73
Creatine	0·28	0·27
Creatinine	0·46	0·46
Protein (heat coagulum)	0·18	0·17

Table 5. *Biological value for the calves of the nitrogen of whole milk at different levels of intake*

Calf no.	Milk intake (l./day)	Apparently digested N (g/day)	Biological value
13	2·6	14·3	40·0
14	3·4	18·3	63·8
14	4·2	22·9	76·6
13	5·0	28·0	70·0
13	5·8	30·4	70·3
14	6·6	35·8	83·6

Respiratory exchange

The respiratory exchange of each calf was determined at the end of each period after the calf had been starved for some 15 h. Table 6 presents the results of these determinations, as well as the mean pulse rates determined throughout the experiment. Statistical analysis by the method previously outlined showed that the regression of heat production per sq.m of body surface per h (HP) on milk ingested in l./day (M) was statistically significant ($P<0.01$). The difference between the calves was not significant ($P=0.12$). The regression equations for each calf were:

calf no. 13 $HP=6.44M+40.1$,
calf no. 14 $HP=6.44M+33.4$,
mean $HP=6.44M+36.8$.

The respiratory quotient was not appreciably affected by the plane of nutrition. The minute volume of the respiration and respiratory rate increased at the higher levels. There was little effect on pulse rate, extremely high values being recorded during the first 2 weeks of the experiment regardless of feeding level. Whether this is an age effect or represents an effect of 'training' is not known.

Table 6. *Respiratory exchanges of the calves*

Calf no.	13	14	14	13	13	14
Milk ingested (l./day)	2·6	3·4	4·2	5·0	5·8	6·6
Time after feeding (h)	14·5	14·0	14·5	14·0	15·5	15·5
Respiratory rate (respirations/min)	26	21	28	43	39	25
Pulse rate (beats/min)*	88	78	112	95	97	106
Minute volume of respiration (l.)	5·8	5·0	7·2	10·9	12·4	9·2
Oxygen consumption (l./h)	13·0	14·1	16·5	18·0	22·2	21·7
R.Q.	0·82	0·80	0·84	0·80	0·75	0·78
Heat production (Cal./h)	63·1	67·6	80·2	86·3	105·1	103·9
Heat production (Cal./sq.m/h)†	56·4	53·6	66·3	70·7	79·6	74·8

* Determined twice daily throughout each period.

† Surface area estimated by the formula: $S = 0{\cdot}15\, W^{0{\cdot}56}$, where S = surface area in sq.m and W = body-weight in kg.

The increase in the intake of milk was clearly associated with a marked increase in the heat production determined 15 h after the previous meal. The increases were large; at a milk intake equivalent to that required for body-weight maintenance the mean heat production per sq.m per h was 53·8 Cal.; when the milk intake was doubled the heat production was 70·9 Cal.; when it was increased three times it was 88·0 Cal. It appears unlikely that this increase was due to unabsorbed milk nutrients remaining in the rumen and abomasum of the calf and reaching the small intestine many hours after the last meal, as it would entail the assumption of a very high heat increment of feeding for what must be very small quantities of milk. The activity of each calf was minimal, so increased muscular activity does not afford an explanation.

Part of the increase may be explained by an increase in muscular tone at the higher nutritional levels, but in any case the results suggest that the heat production of the calf, determined under conditions comparable to those employed in the determination of the basal metabolism of man, is not a stable entity but is sensitive to changes in nutritional plane. That the effect is related to nutritional plane and not to age is shown by the results obtained with calf no. 14. It was 14 days older when given 3·4 l. milk than when given 4·2 l., and its heat production was higher in the earlier period when the nutritional plane was high. Such an instability of basal heat production in relation to nutritional plane has been observed in the rat by Hamilton (1937) and by Treichler & Mitchell (1941). It has also been observed in adult cows by Ritzman & Benedict (1938).

Calcium and phosphorus metabolism

Table 7 summarizes the results of the calcium and phosphorus determinations. Statistically, the regressions of Ca and P balance on milk intake were highly significant ($P < 0{\cdot}01$). In neither case was the difference between calves significant. Where

Ca = the calcium balance and P = the phosphorus balance in g/day, and M = milk ingested in l./day, the regression equations were:

$$\begin{aligned} Ca &= 1{\cdot}408M - 0{\cdot}603, \\ P &= 0{\cdot}857M - 0{\cdot}636. \end{aligned}$$

There was no indication, even at the highest level of intake of milk, that the full capacity of the tissues to retain Ca or P had been reached.

Table 7. *Metabolism of calcium and phosphorus of the calves*

		Ca				P			
			Excretion				Excretion		
Calf no.	Milk ingested (l./day)	Intake (g/day)	Urine (mg/day)	Faeces (g/day)	Balance (g/day)	Intake (g/day)	Urine (g/day)	Faeces (g/day)	Balance (g/day)
13	2·6	3·87	23	1·03	+2·82	2·55	0·80	0·24	+1·51
14	3·4	5·17	37	0·96	+4·18	3·50	0·86	0·26	+2·38
14	4·2	6·26	50	0·47	+5·74	4·11	0·94	0·19	+2·98
13	5·0	7·60	38	0·63	+6·93	5·15	0·94	0·32	+3·88
13	5·8	8·70	40	2·16	+6·50	5·80	1·27	0·75	+3·78
14	6·6	9·90	110	0·71	+9·08	6·60	1·07	0·24	+5·29

The urinary excretion of Ca was low throughout the experiment, amounting to only 5 % of the faecal excretion of Ca. The value is higher than the highest value of 2 % found by Blackwood *et al.* (1936) and does not justify in experiments with calves the omission of the determination of urinary Ca as inferred by these workers.

As previously mentioned, calf no. 13, when given 5·8 l. of milk, scoured badly. The mean daily excretion of ash in the faeces was exceptionally high during this period, as was also the faecal excretion of Ca. The percentage of Ca in the total ash, when the animal scoured, was 27·0 %, which did not differ from the mean percentage of 28·6 found during normal periods. Similarly, the faecal excretion of P increased when calf no. 13 scoured, but the percentage of P in the ash was 9·3 compared with the general mean of 9·6. Thus, although the faecal excretion of ash increased three times during scouring, the proportions of Ca and P in the ash were not affected. This large faecal loss of Ca and P during the alimentary disturbance necessarily resulted in a low retention. The excretion of P in the urine of calf no. 13 increased during the scouring period despite the fact that there was a marked increase in the excretion of P in the faeces. The reason for this is not known; it was certainly not due to an excessive katabolism of body protein during this time. Excluding the results obtained in the scouring period, the increase in urinary P with increasing milk intake was statistically significant ($P<0{\cdot}01$).

DISCUSSION

The effect of nutritional plane on the storage of N, Ca and P, on growth and on heat production, has already been discussed. The two calves, however, differed from one another in several ways. Calf no. 13 required more milk for maintenance of body-weight and for maintenance of N equilibrium than calf no. 14 and it also had a higher basal metabolism. These results are interrelated: a high basal metabolism entails a greater katabolism of ingested food or body tissue to meet this demand for energy, and the higher urinary N largely made up of urea-, ammonia- and amino-N reflects the higher rate of deamination of ingested protein that occurred in order to furnish energy for this purpose. Armsby & Fries (1911) compared the utilization of feed energy by a pure-bred steer with that of a scrub steer and concluded that 'the energy requirement of the scrub steer for maintenance, computed to the same live-weight, averaged 18·7 % higher than that of the pure-bred. Accordingly the latter was able to use a relatively larger proportion of the total energy of his ration for the production of gain.' The difference in heat production of the present calves was 13·2 %, and judging from photographs of Armsby's animals the present calves did not differ in appearance as much as his. The calves in the present experiment were essentially dairy-type animals of the same breed born on neighbouring farms within 1 day of one another and were thought, on purchase, to be comparable in all respects. A further indication of the inherent differences between animals was shown by Wood & Yule (1914), who found that the average live-weight increases of individual oxen on the same diets and under the same conditions could vary between 1 and 3 lb./day. Wood & Hill (1914) later showed that the difference in live-weight increase between 'good doers' and 'bad doers' was inversely related to the skin temperature of the animals, and presumably therefore to their heat production. It is surprising that such large differences exist at such an early age, and these differences must be of considerable practical importance.

From the results presented it is clear that body-weight maintenance in the calf involves storage of minerals in bone and deposition of protein. From the equations presented it can be seen that 2·83 g N, 3·13 g Ca and 1·64 g P were stored daily in this way. The amount of tissue stored in terms of body-weight may be calculated roughly from the Ca, P and N balances. Since the Ca : P ratio in bone is 2·15 (Shohl, 1939), the positive balance of 3·13 g Ca was associated with storage of 1·46 g P. If all the N stored represented a storage of tissue comparable in composition with muscle, 2·83 g N would have been associated with 88 g muscle substance. This protein would have a ratio of N : P of 14·7 (Shohl, 1939) giving an estimate of P retention as muscle of 0·19 g and an estimate of total P retention of 1·65 g against the 1·64 g actually determined. The total gain of body-weight would thus be about 90 g. This gain is presumably balanced by a loss of body fat and possibly by slight dehydration of the tissues.

This general picture of maintenance in the young growing animal is in complete accordance with Waters's (1908, 1910) observations that the body form of cattle kept at constant body-weight did not remain infantile, but that an increase in skeletal proportions and a loss of subcutaneous and depot fat occurred. The results of the present experiment provide a quantitative estimate of these changes in terms of storage of Ca P and N.

SUMMARY

1. Two young calves were given whole-milk diets at levels from 2·6 to 6·6 l./day and their metabolism of nitrogen, calcium, phosphorus and energy was studied.

2. The apparent digestibility of the dry matter, fat and total N of the milk was not affected by the amount given.

3. Both body-weight gain and N storage were linearly related to the intake of milk. Urinary N excretion did not change significantly on increasing the milk intake, and for each additional g of N apparently digested 0·97 g was stored. Biological values increased with increasing milk intake, even at the higher levels of ingestion.

4. The basal metabolism of the calves, 15 h after the last meal, increased from 53·8 Cal./sq.m body surface/24 h at maintenance to 79·5 Cal. at two and a half times the maintenance level.

5. Both Ca and P storages were linearly related to the amount of milk ingested, and at the highest level (about two and a half times the maintenance intake) 92 % of the milk Ca and 80 % of the milk P were retained in the tissues.

6. The two calves differed in their basal energy expenditure at a constant nutritional plane by 13·7 %. The animal with the higher basal metabolism did not grow as quickly or store as much body N as the other and its excretion of urinary N exceeded that of the other by approximately 40 %. This N loss was accounted for by an increased excretion of urea-, ammonia- and amino-N, suggesting a greater deamination of amino-acids in this animal.

7. In confirmation of earlier work it was found that body-weight maintenance did not entail cessation of growth. At body-weight maintenance 2·8 g N, 3·13 g Ca and 1·64 g P were stored daily.

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The Nutrition of the Young Ayrshire Calf

6. The Utilization of the Energy of Whole Milk

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(*Received* 1 *March* 1951)

Two series of experiments with calves to determine the net energy value of whole milk have been published, one in Austria (Soxhlet, 1878) and one in Russia (Tomme & Taranenko, 1939). Soxhlet determined carbon and nitrogen balances in three experiments at a high nutritional plane and, from the results he obtained Armsby, (1917) calculated the net availability of the metabolizable energy of whole milk. To accomplish this Armsby assumed that the maintenance requirement of the calf, per unit of surface area, was 10 % higher than that of the adult steer. He commented that the value he obtained was much lower than would be expected on the basis of the application of Rubner's factors of 69 % for protein, 87 % for fat and 94 % for carbohydrate to the proximate principles present in whole milk. Kellner's (1926) computation of the net energy value of whole milk was, however, considerably lower than Armsby's value, and so indeed was Hansson's (1926). These estimates were made by the application of factors, determined by Kellner on mature cattle, to the proximate principles digested by the calf.

Russian workers (Urmaliat, Reinbot & Elitchiev, 1937; Lebehdiev, 1937) realized these large discrepancies between estimates of the nutritive value of cow's milk, and in 1939 Tomme & Taranenko made six determinations of energy balance with young calves given 7–8 l. of whole milk as the sole diet. They calculated that the starch equivalent of whole milk containing 4 % fat was 21·7, a value higher than Kellner's value of 14·7 and lower than Armsby's value of 27·1. It is uncertain from Tomme & Taranenko's paper what method was used to calculate the maintenance requirement upon which the calculation of net energy ultimately depends. It would appear, however, that fasting heat production determined 12–18 h after the last meal was the basis. Blaxter & Wood (1951 *a*), however, have shown that the heat production of the calf determined at this time is markedly affected by the previous plane of nutrition. There is therefore some uncertainty regarding the nutritive value of the gross energy of whole milk, largely owing to the difficulties involved in determining the basal demand for maintenance energy. For this reason an attempt was made to determine the net energy value of milk by the classical method of measuring the increase in heat production and energy balance that occurs following an increase in food intake.

EXPERIMENTAL

Plan of experiment. An Ayrshire bull calf aged 3 days was used. He was given whole milk only for the 1st week, during which time he became accustomed to the experimental procedure. Unfortunately, after 6 days he had diarrhoea, which was treated by replacing his milk allowance with water for 1 day and by subsequent gradual realimentation. This necessitated a delay in the onset of the experiment which commenced 2 days after the calf was again receiving his whole ration.

The calf was confined in a metabolism cage and harnessed for separate collection of faeces and urine. The technique has already been described by Blaxter & Wood (1951*a*). Two 14-day periods were employed. In the first period the calf was given 4 l. whole milk daily in two feeds. In the second 8 l. were given in three feeds. Four days were taken to effect the change-over, and collection of urine and faeces was not commenced until a further 2 days had elapsed.

Carbon-dioxide determination. The carbon-dioxide production of the calf was determined in a respiration chamber of the open-circuit type. This instrument has been illustrated (Anonymous, 1950) and will be described elsewhere. Briefly, it consists of a metabolism cage (permitting separate collection of excreta) through which a measured stream of outdoor air is drawn. Samples of the air leaving the chamber were taken automatically, and in these samples carbon dioxide and water vapour were determined gravimetrically. The respiratory quotient was not determined accurately. Single samples of outgoing air were taken at hourly, or in some experiments at 2-hourly, intervals, and the oxygen and carbon-dioxide content determined by analysis in a Haldane type apparatus. These were not, of course, true samples, but have been used as rough checks of the heat production determined by carbon- and nitrogen-balance methods. The temperature of the chamber was thermostatically controlled at 20° in the first series of experiments when the calf was given 4 l. milk. In the second series a period of warm weather made it impossible to maintain the temperature at 20° and it rose to 27°. All respiration experiments lasted 24 h. Milk and urine were preserved with mercuric chloride and potassium dichromate.

Analytical methods. The analytical methods for urine and faeces were those previously described (Blaxter & Wood, 1951*b*). Milk fat was determined by the Gerber method. Carbon in urine was determined by wet combustion in a stream of carbon-dioxide-free air, using a solution of silver dichromate in concentrated sulphuric acid to oxidize organic matter. Carbon in the dried faeces, and in the milk previously dried at 70°, was determined by combustion in a tube furnace packed with copper oxide and lead chromate. In either case the carbon dioxide was absorbed by soda lime and weighed. All analyses were made at intervals of 2 days. Three balances of carbon and nitrogen were made when 4 l. milk were given and two when 8 l. were given.

Nitrogen balances were determined throughout but, for the purpose of computing the storage of fat and protein, the balances for the 48 h period, in which the balance of carbon was determined, were employed.

RESULTS

Table 1 shows the method of computation of the energy balances from the balances of carbon and nitrogen. To compute the heat production from these results it is necessary to know the energy intake and the calorific losses in urine and faeces. The energy value of the milk was calculated from the analytical values using the factors of 9·11 Cal./g for fat, 5·86 for protein and 3·95 for lactose (Andersen, 1926). The same factors were used to determine the calorific value of the faeces. The loss of energy in the urine was calculated from its carbon content using a factor of 36·5 Cal./g urinary carbon, a factor calculated from the results of simultaneous determinations of carbon and energy in calf urine by Tomme & Taranenko (1939).

Table 1. *The storage of carbon and nitrogen by the calf during a period when 8 l. milk were given daily*

	Nitrogen (g/day)	Carbon (g/day)
Intake	44·8	535·8
Excretion:		
Faeces	Nil	Nil
Urine	12·8	23·8
Respiration	—	263·4
Storage in body	32·0	248·6
Carbon present in protein	100·9*	
Storage of non-protein carbon	147·7	

Calorie equivalent of nitrogen and non-protein carbon stored

	g	Cal.†
Protein (N × 6·25) stored	200·1	1120
Fat stored	193·4‡	1798
Total	—	2918

* The carbon content of body protein (N × 6·25) was taken as 52·54 % (Kohler, 1901). (N × 3·152 = carbon in protein.)

† The calorific values of body protein (N × 6·25) and fat were taken as 5·6 and 9·3 Cal./g.

‡ The carbon content of body fat was taken as 76·4 % (Schulze & Reineke, 1867). (C × 1·31 = fat.)

In Table 2 the estimation of the heat production is shown. A further estimate of heat production may be made. This involves the computation of the non-protein respiratory quotient, and the use of the Zuntz-Schumburg (1901) tables. As previously stated, oxygen consumption was not as accurately determined as was carbon-dioxide production. Table 2 shows, however, that the agreement between estimates was very good. In the five experiments the maximum difference between the estimates was 118 Cal. in an experiment when 4 l. milk were given. In every case, however, the heat production calculated from the carbon and nitrogen balance was higher than that calculated from the respiratory quotient and oxygen consumption.

Table 2. *Estimation of heat production from the determined energy balance of the calf during a period when 8 l. milk were given daily*

	Cal./24 h	
Milk ingested (calculated from analysis)	6135	
Loss in faeces	Nil	
Loss in urine (calculated from carbon content)	153	3071
Energy of fat and protein stored	2918	
Difference = heat production estimated from carbon and nitrogen balance	3064	
Heat production estimated from oxygen consumption, carbon-dioxide production and urinary nitrogen excretion*	3129	
Difference between estimates of heat production	65	

* See text, p. 14.

Despite this excellent agreement for the individual balances the agreement between balances made at different times was not good. At the high level of nutrition, the balances were 2907 and 3147 Cal./24 h, whereas at the low level they were 742, 1126 and 322 Cal./24 h. This variation was due partly to daily variation in the fat content of the milk, and hence in the energy and carbon content of the milk. In the series at the low level of nutrition, a period in which milk with a low fat percentage of 3·8 and a carbon content of only 6·57 % was given resulted in the lowest balance. Milk with a fat content of 4·3 % and a carbon content of 7·29 % was associated with the highest balance. This, however, was clearly not the sole cause of the discrepancies. At the low plane of nutrition the heat production associated with the lowest balance and milk of the lowest calorific value was higher than any of the other determinations at this level of milk intake. This was related to much greater activity of the calf in the respiration chamber during the period concerned. A series of three 12 h determinations of heat production, lasting throughout the day-time, was then made. The heat production varied from 1400 Cal./12 h on a day when the calf tended to be lethargic, to 1690 Cal./12 h when the calf was jumping in his cage and very active. These results suggest that single determinations of heat production and energy balance in young animals are highly complicated by variation in their activity. This is in accordance with results obtained on human babies (Clements, 1949) in which the activity of crying doubles the heat production.

For the purpose of computation of net energy values, the mean values at each level of feeding have been used, for it was impossible to judge in which experiments activity was the most comparable. The results are given in Table 3.

From the results in Table 3 the net availability of the metabolizable energy may be computed using the equation:

$$\text{energy balance} = a \times \text{metabolizable energy} - b \times \text{metabolic body size},$$

where a represents net availability, and $b \times$ metabolic body size represents maintenance requirements. Metabolic body size is the interspecies unit of basal metabolism represented by body-weight raised to the power 0·73 (Brody, 1945). Using simultaneous equations, the value of a was found to be 0·845, and of b 121·6. The net availability of the metabolizable energy of whole milk was thus 85 %, and the maintenance requirement about 120 Cal. of net energy or 140 Cal. of metabolizable

Table 3. *Mean body-weight, daily gain of weight, metabolizable energy ingested and energy balance of the calf at two planes of nutrition*

	Milk intake	
	4 l./day (mean of three experiments)	8 l./day (mean of two experiments)
Mean body-weight (kg)	35·1	46·7
Mean daily gain of body-weight (g)	390	950
Metabolizable energy* (Cal.)	2801	5969
Energy balance† (Cal.)	730	3031
Metabolic body size‡ (kg)	13·44	16·54

* Gross energy ingested less energy of the excreta.
† Determined from carbon and nitrogen balances.
‡ From Brody's (1945) formula: metabolic body size = $W^{0\cdot73}$, where W = body-weight in kg.

energy/kg body-weight raised to the power 0·73. For a calf weighing 35 kg ($35^{0\cdot73}$ kg = 13·4 kg) this would be equivalent to 1608 Cal. of net energy or 1876 Cal. of metabolizable energy per day. These estimates may be compared with previous estimates made by regression analysis of body-weight changes in calves where the maintenance requirement of the 30–35 kg calf was found to be 1771 ± 97 Cal. digested energy/day and in other trials 1921 ± 129 Cal. (Blaxter & Wood, 1951 *c*, 1952). Digested energy, less the small amount of energy in the urinary solids, is the metabolizable energy. These three estimates do not differ markedly from one another. All are very much higher than either the interspecies normal basal metabolism of adult animals of 70·4 Cal. of net energy/kg body-weight raised to the power 0·73 (Brody, 1945) or the minimum base value of heat production of mature cattle and sheep, which is approximately 50 Cal. net energy/kg metabolic body size (Forbes, Braman, Kriss & Swift, 1931; Forbes & Kriss, 1932; Marston, 1948). This emphasizes both the intensity of metabolism in the young, active, growing animal, and the errors inherent in Armsby's assumption that the maintenance requirement of the young is but slightly greater per unit of surface than that of the adult.

The total energy ingested was metabolized to the extent of 94 % at the lower level of feeding and of 97 % at the higher, the loss of energy in the faeces being the main determinant. A figure of 95 % has been taken as representative of the normal animal.

DISCUSSION

It is of interest to compare the present results with those of Soxhlet (1878) and of Tomme & Taranenko (1939). This is done graphically in Fig. 1, where the metabolizable energy and the energy balance have been expressed on the basis of metabolic body size ($W^{0\cdot73}$) following the method of Marston (1948). It will be seen that the spread of the values about the mean line is considerable. Part of this is due to individual differences between the calves, but from the results obtained in the present study a large part is thought to be due to variation in heat loss caused by the activity of the calf in the confines of its cage. The line passing through the distribution is one obtained using the maintenance value of 144 Cal. metabolizable energy and a slope of

0·85, as obtained in the present study. It will be noted that whereas Soxhlet's results agree admirably, the Russian results mostly fall below the line, suggesting that these calves had greater maintenance requirements than either the Austrian or British calves. This may be a breed difference or an activity difference or an effect of body size, for the Russian calves were much larger than the others. In any case, indiscriminate pooling of the data is not warranted, and despite the large variation in conditions the results are in good agreement.

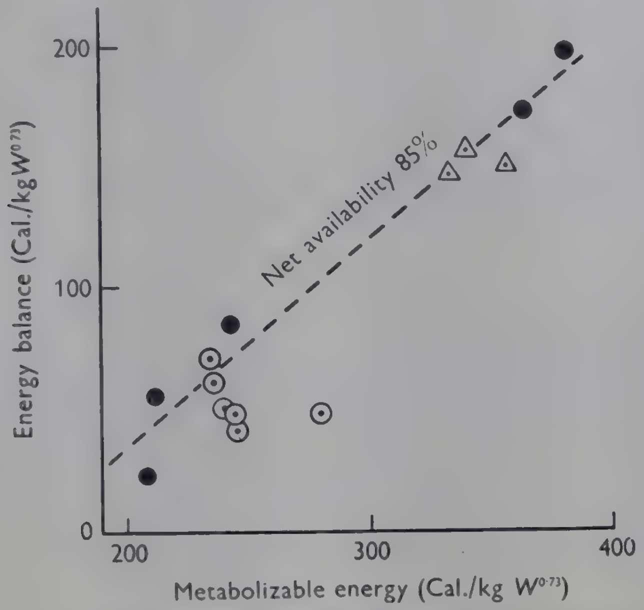

Fig. 1. The relationship between the intake of metabolizable energy and the balance of energy both expressed as Cal./kg body-weight raised to the power 0·73 in Soxhlet's (1878), Tomme & Taranenko's (1939) and the present experiments with calves. ●, present results (1951); △, results of Soxhlet (1878); ⊙, results of Tomme & Taranenko (1939).

Previous estimates of the metabolizable energy value or starch equivalent of milk may now be examined. Table 4 summarizes the calculations. The estimates are of two types: those based on direct experimentation with calves in which energy balances have actually been determined and those based on the application of factors to the digested components of the diet. Of the former group, both Armsby's and Tomme &

Table 4. *The net energy value of whole milk containing 4 % fat and 753 gross Cal./kg*

Method of calculation	Gross energy metabolized (%)	Net availability of metabolizable energy (%)	Net energy/kg. (Cal.)
Armsby's calculations from Soxhlet's (1878) results	95·0	73·8	528
Tomme & Taranenko's (1939) experimental results	92·2	74·2	512
Present experimental results	95·0	85·0	608
Using Kellner's factors for ruminants*	95·0	56·6	405
Using Hansson's factors for ruminants*	95·0	62·3	446
Using Rubner's factors for simple-stomached animals†	95·0	84·3	603

* These are given in full by Blaxter (1949–50).

† See text, p. 12.

Taranenko's results involve the assumption of a maintenance base-line. The present estimates are free from such assumption, and, as shown in Fig. 1, the energy balances observed by Soxhlet and, to a lesser extent, those of the Russian workers, agree quite well with the present calculation. The estimates based on factors, however, show very large discrepancies. Both Kellner's (1926) and Hansson's (1926) factors were based on values for the energy exchanges of mature steers. In these animals the heat increment due to food is very large and the net availability of metabolizable energy consequently small. The intermediary metabolism of the lower steam-volatile fatty acids which are produced in large amounts by microbial activity in the rumen and caecum is thought by most workers to be the main reason for this high heat increment (Phillipson, 1947–8). It would be expected, therefore, that these factors would considerably underestimate the net energy value of food in an animal in which extensive fermentation does not take place. The low net energy values given by both Kellner's and Hansson's factors can thus be attributed to the assumption that the assimilation of food materials in the non-ruminant calf involves the same energy loss as in the ruminant adult. Rubner's factors, however, were determined by calorimetric experiments with dogs and men and not with ruminating animals. The estimate they provide of the net energy value of milk is in close agreement with the experimental results for calves. It may be concluded therefore that their application to diets given to the calf before rumen development occurs is justified.

SUMMARY

1. Five carbon and nitrogen balances have been determined with a calf given cow's whole milk as its sole diet at two planes of nutrition.

2. It was found that activity of the calf within the confines of its cage was an important variable in determination of energy storage.

3. The energy balances were used to compute the net availability of the metabolizable energy of cow's whole milk, and gave a value of 84·5 %.

4. The maintenance requirement of the calf per unit of metabolic body size was 122 Cal. of net energy or 144 Cal. of metabolizable energy. This value was in agreement with estimates made by another method.

5. The results show that Rubner's factors may be used to compute the net energy value or starch equivalent of calf foods for calves that have not started ruminating.

My grateful thanks are due to Mr D. Paterson for his assistance in the 24 h experiments and in the calibration and maintenance of the respiration apparatus.

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The Assessment of the Growth of Schoolchildren with Special Reference to Secular Changes*

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(*Received* 9 *March* 1951)

An interesting phenomenon, prominent in the present century, is a secular change in the pattern of growth of schoolchildren. It is usually regarded simply as an increase in the average height and weight of children of school age. This aspect, however, may be misleading and is not altogether satisfactory for comparative purposes. The change was therefore investigated, first in terms of age of attaining a given height or a given weight, and then in terms of rate of growth. This approach leads to the view that the secular change in the pattern of growth is essentially an acceleration.

METHODS AND RESULTS

Data surveyed

The figures dealt with here have been confined to the measurements of boys in Glasgow and London and two sets of data for England as a whole. In 1905–6 a very extensive survey was made of the heights and weights of Glasgow schoolchildren, the occupation of their parents, the number of rooms occupied and other similar information. The schools from which the children came were divided into four groups.

Group A comprised schools in the poorest districts of the city, group B in poor districts of the city, group C in better-class districts and group D in districts of a still higher class, including four out of five Higher Grade Schools.

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* An abridged version of this paper was read at the sixty-third scientific meeting of The Nutrition Society on 14 October 1950, the topic under discussion being *Growth.*

Some of these data were later analysed by Miss Elderton (Elderton, 1914) and her paper gives a very complete picture of the heights and weights of Glasgow schoolchildren at that time. Average heights and weights of Glasgow schoolchildren from 1910 to 1949 are available in a series of annual reports by the Education Health Service of the Corporation of Glasgow (see, for example, Ewan, 1950; Young, 1945). The measuring was suspended during the first World War and also in 1940. The figures are otherwise complete for three age groups only, namely 5, 9 and 13 years.

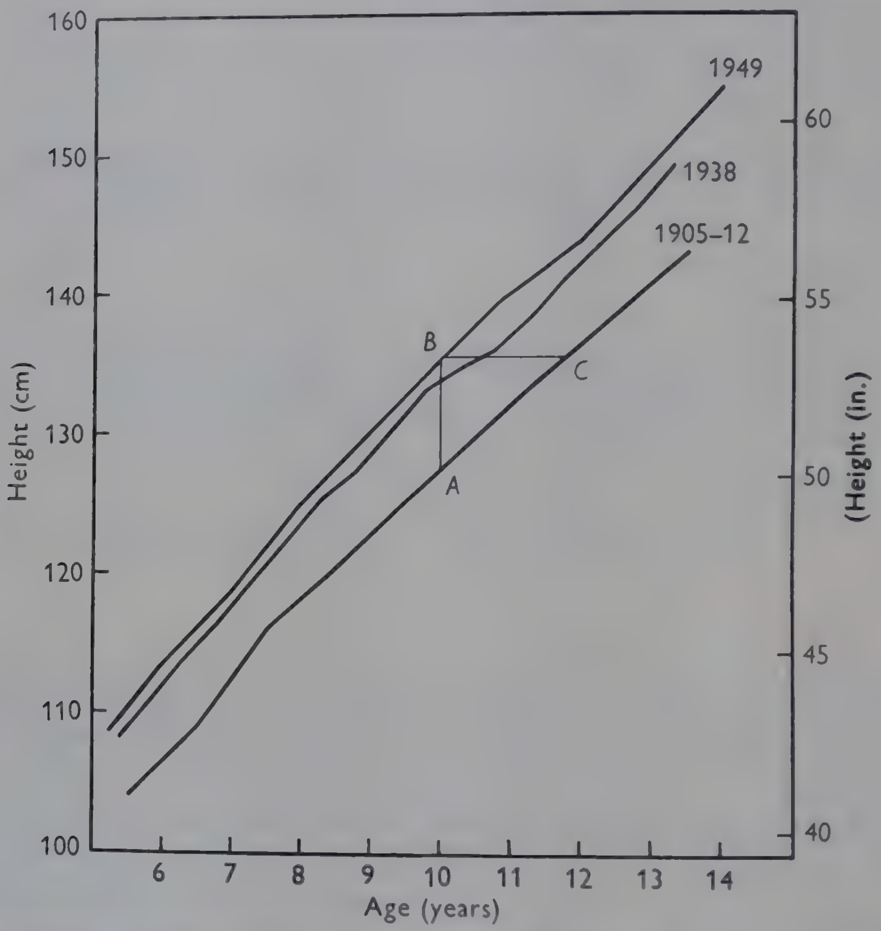

Fig. 1. Secular changes in growth of London schoolboys. Two points of view are indicated; *AB* represents the increase in height at a given age and *BC* the earlier attainment of a given height.

The measurements of London schoolchildren in the years 1905–12, 1938 and 1949 are given in two London County Council Reports (Menzies, 1940; Daley, 1950). The former also quotes some data for English elementary schoolchildren in 1909 and in 1927. All five sets of data cover the age groups 5–14 years.

London measurements

The relation of the average heights of London schoolboys to age is shown in Fig. 1 for the years 1905–12, 1938 and 1949. The corresponding curves for weight are shown in Fig. 2. It is clear that there has been a considerable increase in both height and weight between 1905–12 and 1938, that the process has continued to 1949 and that it applies to all ages. The increase in height appears approximately constant at all ages, but it is evident that the increase in weight is progressive with age. The increases in the measurements adjusted as described below (p. 24) to the stated ages are given in Table 1. The changes in height do in fact increase somewhat with age. The 1905–12 to 1938 interval suggests a decline through the age groups in the percentage increase of height, but the longer interval 1905–12 to 1949 shows that it is almost constant at all ages. The actual increases in weight are definitely progressive and even the percentage increase shows an upward tendency.

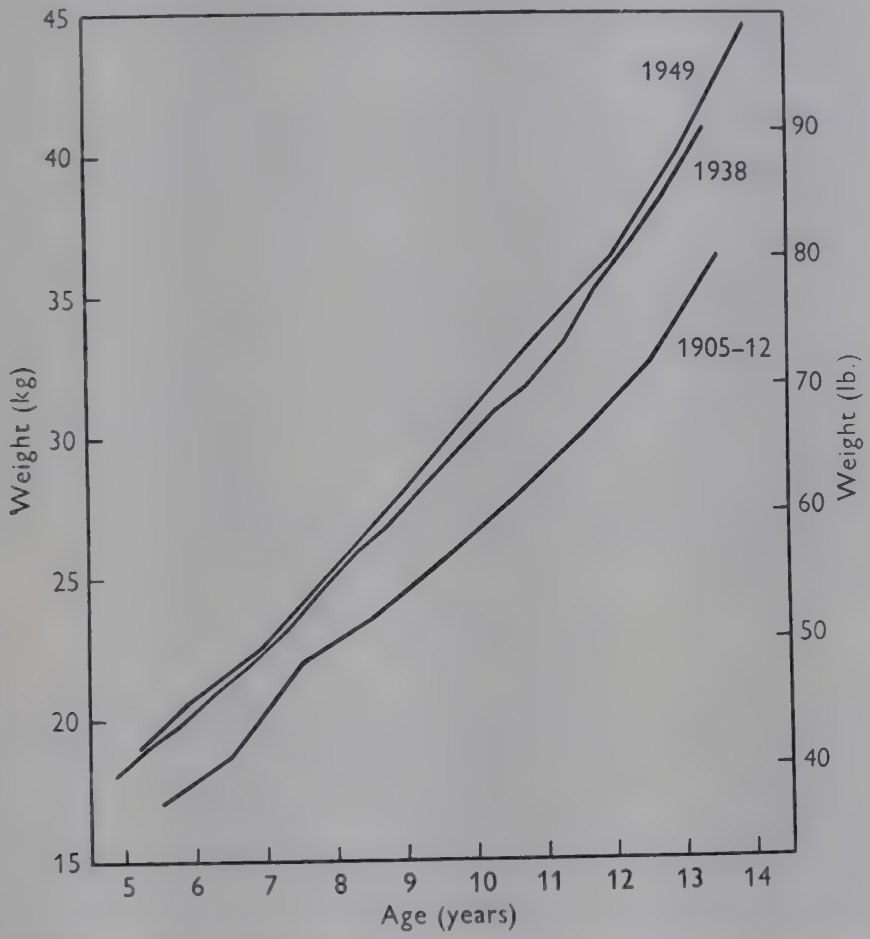

Fig. 2. Secular changes in growth of London schoolboys. Relation of weight to age.

Table 1. *Secular changes in average height and weight of London boys (modified from Menzies,* 1940, *Table V and Daley,* 1950, *Table* 4)

	Height						Weight					
	Excess of 1938 over 1905–12		Excess of 1949 over 1938		Excess of 1949 over 1905–12		Excess of 1938 over 1905–12		Excess of 1949 over 1938		Excess of 1949 over 1905–12	
Central age (years)	(cm)	(%)	(cm)	(%)	(cm)	(%)	(kg)	(%)	(kg)	(%)	(kg)	(%)
5·5	5·1	4·9	1·4	1·28	6·5	6·24	2·4	14·1	0·23	1·19	2·6	15·2
6·5	5·9	5·4	1·3	1·12	7·2	6·60	2·7	14·4	0·25	1·17	3·0	15·9
7·5	4·5	3·9	1·5	1·24	6·0	5·17	1·7	7·7	0·37	1·63	2·0	9·2
8·5	5·3	4·4	1·7	1·35	7·0	5·80	2·6	11·1	0·65	2·49	3·2	13·7
9·5	5·7	4·6	2·0	1·53	7·7	6·15	3·2	12·5	0·84	2·93	4·0	15·7
10·5	6·0	4·6	2·1	1·55	8·1	6·23	3·9	14·1	0·87	2·77	4·7	17·1
11·5	6·1	4·5	1·6	1·14	7·7	5·73	4·5	15·1	0·57	1·65	5·1	17·1
12·5	6·1	4·4	1·7	1·17	7·8	5·62	5·1	15·7	0·68	1·80	5·8	17·9
13·5	5·9	4·1	3·2	2·15	9·1	6·35	4·7	12·9	1·71	4·16	6·4	17·6
Mean	5·62	4·53	1·83	1·39	7·46	5·99	3·42	13·7	0·67	2·20	4·09	15·5

Glasgow measurements

The Glasgow data shown in Figs. 3 and 4 have the advantage that the time span is more completely covered, and show the trend of the secular change over a period of 40 years. It will be noted that, whereas the trend is upwards throughout the period, the gradient has not been uniform. Little change took place till after the first World War and the subsequent economic depression. The yearly data (not shown) also suggest a deceleration during the depression in the early thirties. There was a marked increase in 1941. The reasons for this are complex. During the previous year a big exodus of schoolchildren took place from the city, followed by the return of most of the evacuees. Periods of evacuation would benefit many of the children. The increase is larger than could be accounted for by the evacuation of the more stunted children and omission of their measurements from the totals. In fact there was a greater frequency of sustained evacuation among older girls from secondary schools in certain better-class areas of the city and probably among the better pupils in other groups. Young (1942) summed up the position as follows: 'To what extent increased family incomes, the milk in schools scheme, the absence of major epidemics of infectious diseases and perhaps periods of evacuation have contributed to this result it is difficult to say.' For the next few years there was a lag in growth, especially in height, but by 1949 the measurements exceeded even the exceptional figures of 1941.

The assessment of growth

In assessing any change over a period of years the accuracy of the measurements must be considered. It is becoming appreciated nowadays that routine measurements of heights and weights are subject to appreciable errors of observation. The variance from this source, however, is small in comparison with the total variance. Systematic errors may arise from differences in the clothing conditions under which the measurements are made. All the data surveyed here, as far as can be ascertained, refer to 'indoor clothing without shoes'. In a small survey the number of individuals in each sample group is of critical importance and should always be recorded. Thus even in the 1949 London survey, based on measurements of 21,000 children, the standard errors of the age groups means approximate to 0·2 cm in height and from 0·09 to 0·25 kg in weight. Both these standard errors exceed the average secular yearly change.

A serious problem, curiously enough, is that of the age. Age is generally regarded as the independent variable and assumed to be accurate; but, apart from gross errors such as that of confusing central age and age last birthday (Elderton, 1914), it is usually subject to a sampling error. If the age last birthday at the time of measurement is used, then on an average the mean age of each group will be age last birthday plus 6 months. This expected difference of 6 months is associated with a standard error of $1/\sqrt{(12n)}$ years (where n is the number in the sample) which can if desired be converted into an equivalent error of height and weight. In practice it is convenient to classify the children according to year of birth rather than age last birthday, and the raw age groups are then year of observation less year of birth. The mean age of

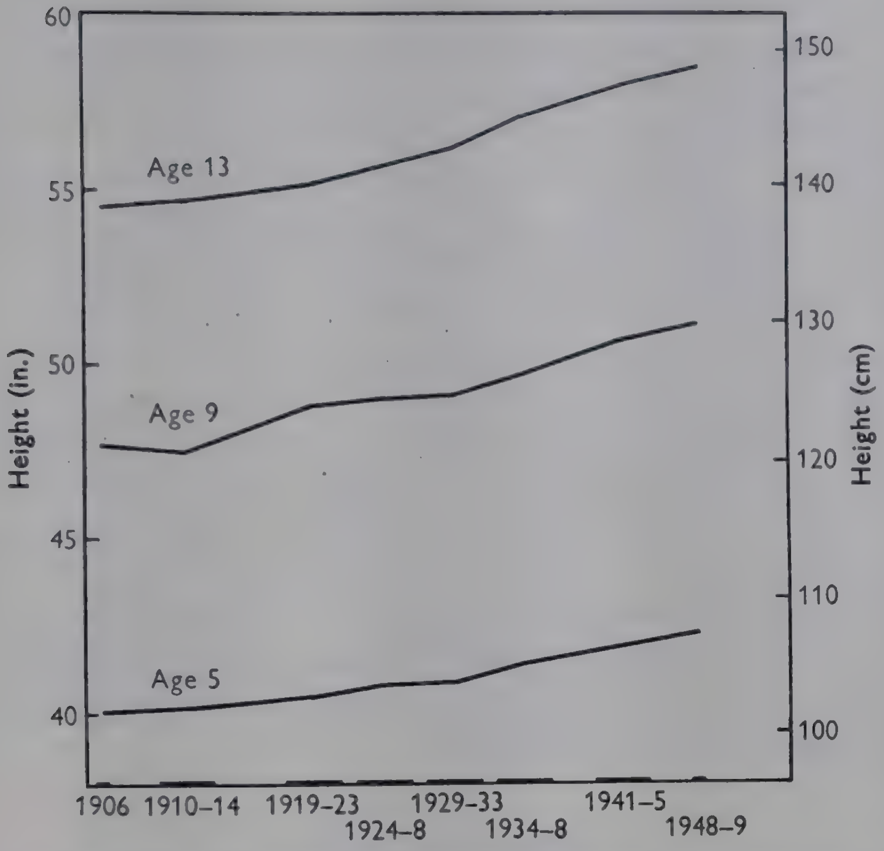

Fig. 3. Secular changes in growth of Glasgow schoolboys. Average values of height of age groups 5, 9 and 13 years for the periods indicated.

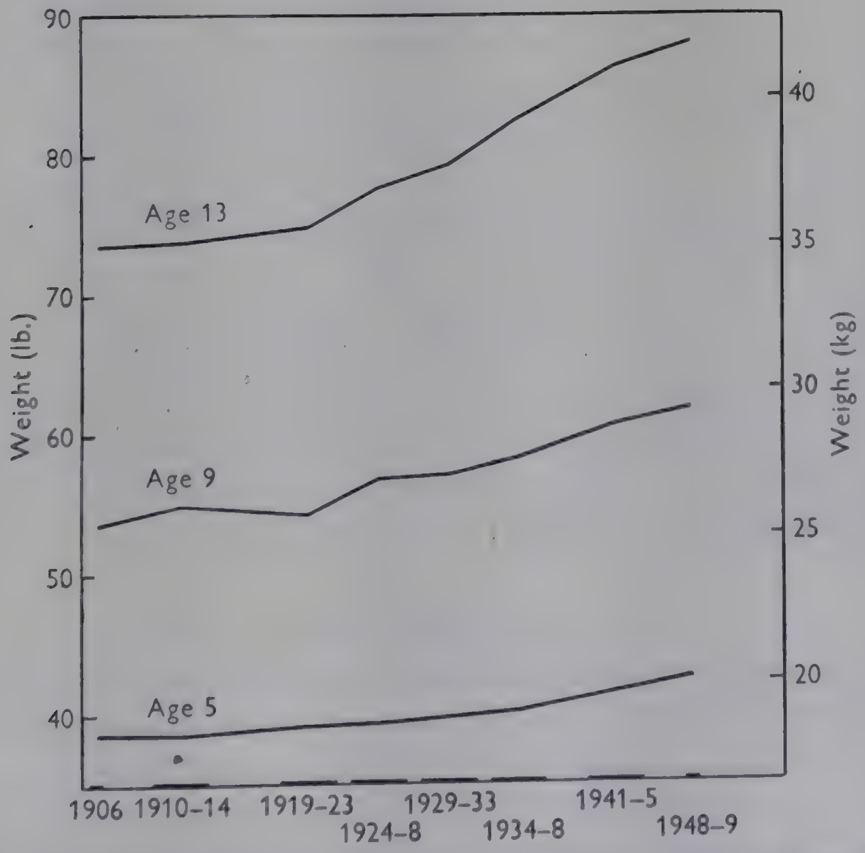

Fig. 4. Secular changes in growth of Glasgow schoolboys. Average values of weight of age groups 5, 9 and 13 years for the periods indicated.

each group will of course depend on the time of year at which the measurements are taken and is subject to the above sampling error with an additional sampling error in the mean date of measurement. There is thus an element of uncertainty as to the exact mean age of the various groups from different sources of data.

A further difficulty is that in presenting the measurements it is customary to adjust them to certain fixed ages. In the 1949 London data this was done by linear interpolation, whereas in the 1938 figures parabolic curves were fitted to the raw data, and heights and weights then calculated at 3-month intervals. In Glasgow the main age groups have standard central ages of 5 years 4 months, 9 years 5 months and 13 years 5 months. Adjustment to these is effected by using the regressions of height and weight on age. The estimates of these regressions have varied considerably.

The net result then is that the heights and weights at stated intervals obtained from a variety of sources covering a matter of 40 years are subject to appreciable errors which are probably mainly random and so would account for many of the deviations from smooth curves in the accompanying figures. Fortunately, the secular changes over a period of years are quite large in comparison with the total estimated error so that the main features are beyond doubt, but this lack of precision is a drawback in the more detailed analysis of the changes.

Reduction of data

Surveys of heights and weights produce very large numbers of figures and to compare one survey with another presents a difficult problem. An obvious step is to reduce the data to the means at each age group and then, as in Table 1, to compare the means in corresponding groups. A further step is to compare the averages over all the age groups, provided that they correspond, or at least that the mean age is the same. An example of the expression of the result in this form is that Glasgow boys were on the average 3·21 in. taller and 8·24 lb. heavier in 1949 than in 1910–14. What is the significance of these differences? The higher age groups contribute more to them than the lower groups; the regression of difference of height on age is probably curvilinear and that of weight on age definitely curvilinear. At the best it gives some kind of indication of the difference between an average child in the central age group of one survey and a similarly situated child in the other. The difference is less in the lower age groups and more in the higher. An improvement can be effected by using either the logarithms of the heights and weights or the percentage changes, but the method developed below is preferable.

Units of growth

The relationship between changes in height and simultaneous changes in weight can be studied directly; but it seemed that it would be illuminating and also more convenient to use the common link between height and weight, namely age, and to study the changes in terms, not of height and weight, but of a year's growth. Fig. 1 indicates that the secular change in growth, besides being regarded as an increase in the average height and weight of each age group, may equally well be considered as an earlier attainment of the same average measurement, in other words as more rapid growth. Fig. 5 shows the London 1938 smoothed weights plotted against the smoothed heights and provides a standard track or axis of growth. Any deviation from the track implies a difference of form or build from the standard. Now, when any of the other sets of average heights and weights are plotted on the same diagram it is found that the points lie very close to this standard track but are advanced or retarded with reference to the age scale. The main effect of the secular change is more rapid growth, and any change of form is subsidiary. In view, however, of the possibility of different effects on the rate of growth in height and weight it was decided to keep them separate and to use two units of growth, a year's growth in height and a year's growth in weight.

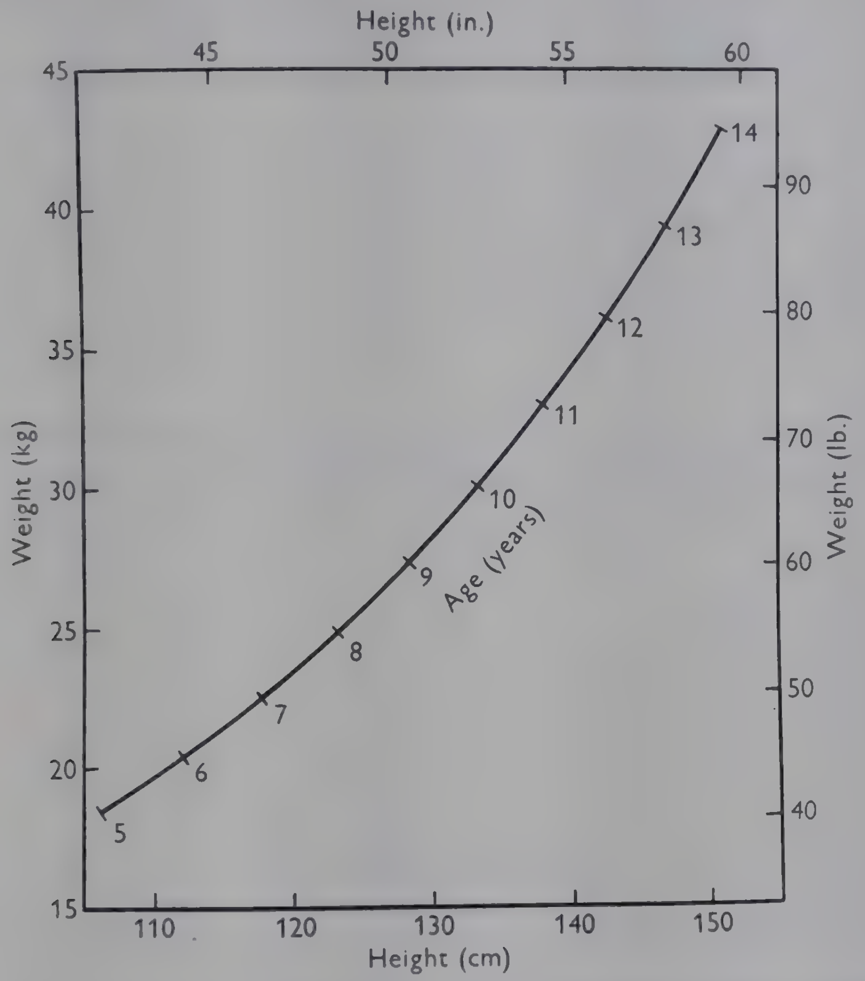

Fig. 5. Standard growth track. Relation of average height and weight of 1938 London schoolboys to age in years.

Ages for height and for weight—growth ages

A diagram on the lines of Fig. 5 is adequate for many comparisons, but to obtain greater precision parabolic curves were fitted by the method of least squares to the 1938 London data which give age in terms of height and age in terms of weight. Tables 2 and 3 were then drawn up. Table 2 gives the age to the nearest 0·01 year for each cm of height from 100 to 150 cm and mean differences for mm. Table 3 similarly gives ages corresponding to each 0·1 kg from 17 to 41 kg and mean differences for

0·01 of 1 kg. With the aid of the tables the age corresponding to any height or weight growth-age within the range is readily found, and comparison with the chronological age indicates at once the relative advancement or retardation in terms of year's growth.

Table 2. *Age in years corresponding to height in cm in boys (based on average heights of London boys: data of Menzies, 1940)*

Height (cm)	cm										Mean differences for mm hei						
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7
100	4·03	4·18	4·34	4·50	4·66	4·82	4·98	5·15	5·31	5·48	2	3	5	6	8	10	11
110	5·65	5·82	5·99	6·16	6·34	6·51	6·69	6·87	7·05	7·23	2	4	5	7	9	11	12
120	7·42	7·60	7·79	7·98	8·17	8·36	8·55	8·75	8·95	9·14	2	4	6	8	10	12	13
130	9·34	9·54	9·75	9·95	10·16	10·36	10·57	10·78	10·99	11·21	2	4	6	8	10	12	15
140	11·42	11·64	11·86	12·08	12·30	12·52	12·74	12·97	13·20	13·42	2	4	7	9	11	13	16
150	13·66	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 3. *Age in years corresponding to weight in kg in boys (based on average weights of London boys: data of Menzies, 1940)*

Weight (kg)	0·1 kg										Mean differences for 0·01 kg w						
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7
17	4·33	4·38	4·43	4·48	4·53	4·58	4·63	4·68	4·73	4·78	1	1	2	2	3	3	4
18	4·83	4·88	4·93	4·98	5·03	5·08	5·13	5·18	5·23	5·28	0	1	1	2	2	3	3
19	5·32	5·37	5·42	5·47	5·52	5·57	5·62	5·66	5·71	5·76	0	1	1	2	2	3	3
20	5·81	5·85	5·90	5·95	6·00	6·04	6·09	6·14	6·18	6·23	0	1	1	2	2	3	3
21	6·28	6·32	6·37	6·42	6·46	6·51	6·55	6·60	6·64	6·69	0	1	1	2	2	3	3
22	6·74	6·78	6·83	6·87	6·92	6·96	7·01	7·05	7·10	7·14	0	1	1	2	2	3	3
23	7·18	7·23	7·27	7·32	7·36	7·40	7·45	7·49	7·53	7·58	0	1	1	2	2	3	3
24	7·62	7·66	7·71	7·75	7·79	7·84	7·88	7·92	7·96	8·01	0	1	1	2	2	3	3
25	8·05	8·09	8·13	8·17	8·22	8·26	8·30	8·34	8·38	8·42	0	1	1	2	2	2	3
26	8·46	8·50	8·54	8·59	8·63	8·67	8·71	8·75	8·79	8·83	0	1	1	2	2	2	3
27	8·87	8·91	8·95	8·99	9·03	9·07	9·11	9·14	9·18	9·22	0	1	1	2	2	2	3
28	9·26	9·30	9·34	9·38	9·42	9·45	9·49	9·53	9·57	9·61	0	1	1	2	2	2	3
29	9·64	9·68	9·72	9·76	9·79	9·83	9·87	9·91	9·94	9·98	0	1	1	1	2	2	3
30	10·02	10·05	10·09	10·13	10·16	10·20	10·23	10·27	10·31	10·34	0	1	1	1	2	2	2
31	10·38	10·41	10·45	10·48	10·52	10·55	10·59	10·62	10·66	10·69	0	1	1	1	2	2	2
32	10·73	10·76	10·80	10·83	10·86	10·90	10·93	10·97	11·00	11·03	0	1	1	1	2	2	2
33	11·07	11·10	11·13	11·17	11·20	11·23	11·26	11·30	11·33	11·36	0	1	1	1	2	2	2
34	11·39	11·43	11·46	11·49	11·52	11·55	11·59	11·62	11·65	11·68	0	1	1	1	2	2	2
35	11·71	11·74	11·77	11·80	11·84	11·87	11·90	11·93	11·96	11·99	0	1	1	1	2	2	2
36	12·02	12·05	12·08	12·11	12·14	12·17	12·20	12·23	12·26	12·28	0	1	1	1	1	2	2
37	12·31	12·34	12·37	12·40	12·43	12·46	12·49	12·51	12·54	12·57	0	1	1	1	1	2	2
38	12·60	12·63	12·65	12·68	12·71	12·74	12·76	12·79	12·82	12·84	0	1	1	1	1	2	2
39	12·87	12·90	12·92	12·95	12·98	13·00	13·03	13·06	13·08	13·11	0	1	1	1	1	2	2
40	13·13	13·16	13·19	13·21	13·24	13·26	13·29	13·31	13·34	13·36	0	1	1	1	1	2	2
41	13·39	13·41	13·43	13·46	13·48	13·51	13·53	13·56	13·58	13·60	0	0	1	1	1	1	2

It should be noted that the tables give the age at which the London boys attained given average heights and weights, and do not necessarily represent the average age of attaining a given height and weight. There is no longer any necessity to adjust measurements to fixed ages before they can be compared. The tables can also be used inversely to give the standard height and weight corresponding to a given age. The values so found do not differ significantly from the regressions of height and of weight on age obtained directly. The use of the tables is not confined to comparisons with the

London 1938 averages since they can be used simply as a yardstick to compare two or more individuals or groups.

Relation of growth age to chronological age

A matter of some importance is the relation of growth age to age or of advancement or retardation to age. Menzies (1940) comparing London children in 1938 with those in 1905–12 finds that the advancement is approximately constant at 1 year's growth. However, when the deviations of each set of data from the standard are examined it is evident that they are progressive with age. The trend is quite clear when the deviations are plotted against age as in Fig. 6. All the sets of data show some degree of irregularity, but the regressions show no consistent deviation from linearity. In some instances the points agree best with a straight line which meets the age axis somewhat to the negative side of the origin, but on the whole for the range studied all agree reasonably well with straight lines through the origin, and this is a great practical advantage. On this basis the secular change may be regarded as a uniform decrease in the scale factor, for time and growth age may be regarded as directly proportional to chronological age. The deviations of the points from straight lines—residual error—are partly due to the sampling and other errors already discussed and partly to irregularities in the secular change itself and to imperfections in the standard. The secular change has varied from time to time with wars, depressions and similar causes. It will be appreciated that the physique of the 5-year-old children is an index of conditions over the previous 5 years, that of the 10-year-old children an index of the previous 10 years and so on. Thus what is compared is one composite group of years with another similar group some years later, and variations in the secular change will affect the different age groups to different extents.

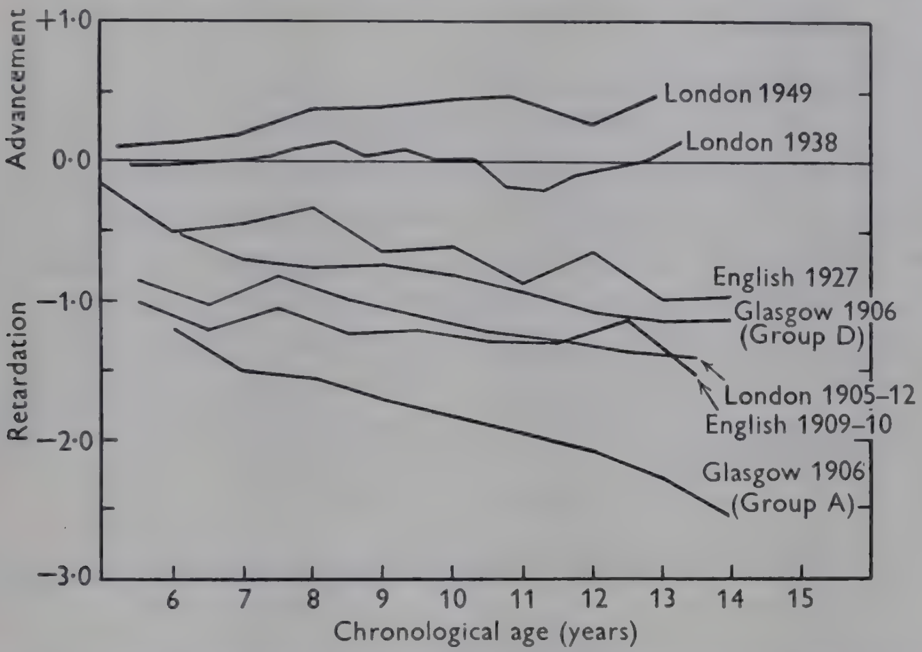

Fig. 6. Relation of relative advancement or retardation of growth in height (height age less chronological age) to chronological age. The retardations or advancements are progressive and approximately proportional to age. Glasgow 1906 (group A) poorest districts, Glasgow 1906 (group D) better class districts.

Growth-rate indices

When the age for height and age for weight, whether of an individual or of an age group, are expressed as percentages of the chronological age they give the growth rate as a percentage of the standard growth rate and so form convenient indices of growth, which will be called the growth rate in height and the growth rate in weight. In so far as the growth ages are proportional to chronological ages the indices are independent of age. This is confirmed by actual calculation, though in some instances in which the indices are less than 100 they increase slightly with age.

Estimate of group indices

The comparison of the growth of groups of children of various ages is greatly simplified by the use of a growth-rate in height index and a growth-rate in weight index for each group. These group indices are essentially weighted means of the individual or subgroup indices. Assuming that the growth ages within each group, apart from random variations, are proportional to the chronological ages, the calculation of a group index is equivalent to that of finding the gradient of the 'best' straight line through the origin in the plot of growth age against chronological age. There is a choice of three methods. Let x years be the chronological age, y years the growth age (in height or weight), b the gradient or group index and n the number of points. Then:

(1) Group index $= b = \dfrac{\Sigma(y/x)}{n}$ or mean value of y/x;

(2) $b = \Sigma y/\Sigma x$; hence the group index = mean growth age divided by mean chronological age;

(3) $b = \Sigma xy/\Sigma x^2$.

With the data examined the differences in the results by the three methods are not significant. The second method is the simplest when only the index is required and is the method used for the results given in Table 4 and plotted in Fig. 9. Ideally the method yielding the minimum variance should be chosen, but simplicity of calculation may be more important.

Chronological age multiplied by the group index gives the regression of growth age on chronological age or the expected growth age for each member of the group.

Combination of growth curves

Any set of data forming the basis of a growth curve is subject to sampling and other errors. The question therefore arises: can several sets of data, e.g. the three sets of average weights shown in Fig. 2, be combined to give a more reliable and representative curve? To do this the secular change or other difference of a similar nature must be eliminated. In Fig. 7 the same data as in Fig. 2 have been plotted against expected growth age instead of against chronological age. All three sets of data now agree satisfactorily with each other thus affording a neat confirmation of the validity of the assumption of proportionality of growth age and chronological age. A cubic parabola might give a slightly better fit at the extremes, but the curve shown (simple parabola) which was fitted to the 1938 data appears to be adequate for this range.

Secular change and social conditions

The secular change in growth is very similar to the variations that are associated with differences in social conditions. Table 4 gives the growth-rate indices corresponding to all the data dealt with here. It will be noticed that the secular change over a period of some 40 years has considerably exceeded the range of the 1906 Glasgow groups. The comparison is elaborated in the Glasgow report for 1944 (Young, 1945) which gives the average heights and weights of the standard age groups classified according to the number of apartments* in the house. The heights and weights of the schoolchildren increase with the number of apartments; in all groups there are increases from 1906 to 1944; the increases are greatest for the single apartments. In

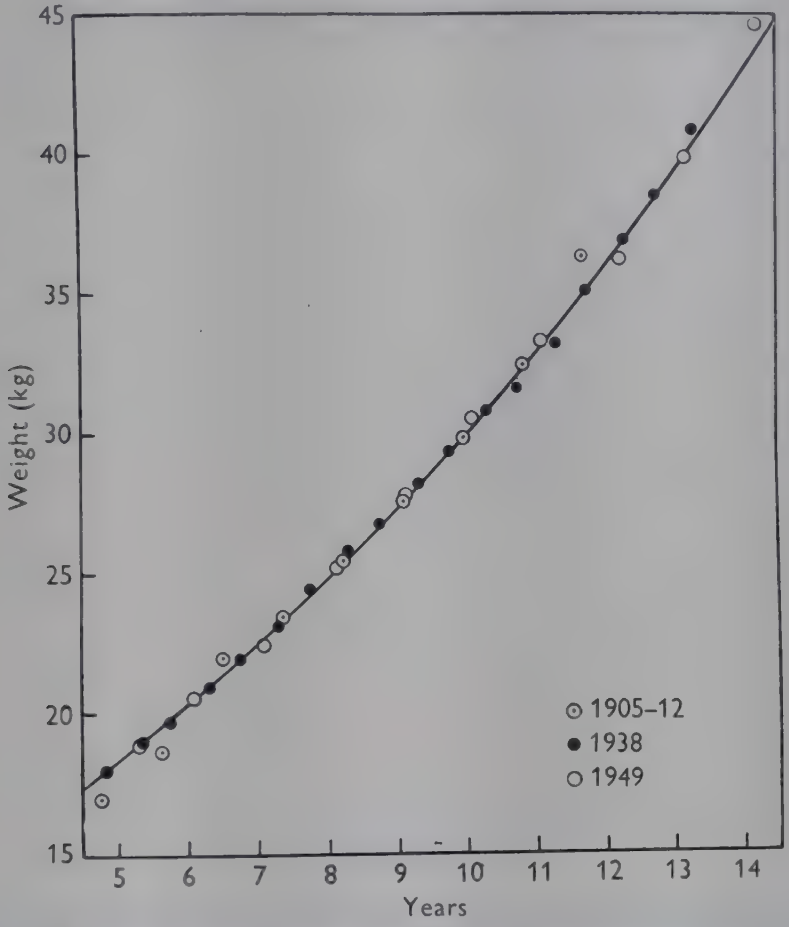

Fig. 7. Combination of growth curves. Average weights of London schoolboys in 1905–12, 1938 and 1949 plotted against *expected growth ages*, i.e. age × 0·866 for 1905–12 group, age × 1·0 for 1938 group and age × 1·0215 for the 1949 group. The curve was fitted to the 1938 values. Compare with Fig. 2.

Table 4. *Growth-rate indices of height and weight of London, English and Glasgow boys (estimated by method 2, p. 28) for the years and social groups indicated. The 1938 London average is taken as the standard and is 100 for both indices*

	London		English		Glasgow				Glasgow 1906 groups			
ndex	1905–12	1949	1909–10	1927	1910–14	1927	1938	1949	A	B	C	D
eight	88·3	103·9	87·6	93·6	82·0	89·1	93·8	99·1	81·5	84·6	87·3	91·4
eight	86·6	102·2	86·2	89·7	84·5	88·7	93·7	98·5	83·5	86·1	88·3	90·8

* 'Apartments' as given here is equivalent to the English usage of 'rooms'.

the 5-year age group the single-apartment figures for 1944 are comparable with the best 1906 figures. In the 9-year age group the 1944 figures for single apartments are considerably better than the best 1906 figures. The 13-year age group similarly shows a superiority over the best 1924 figures.

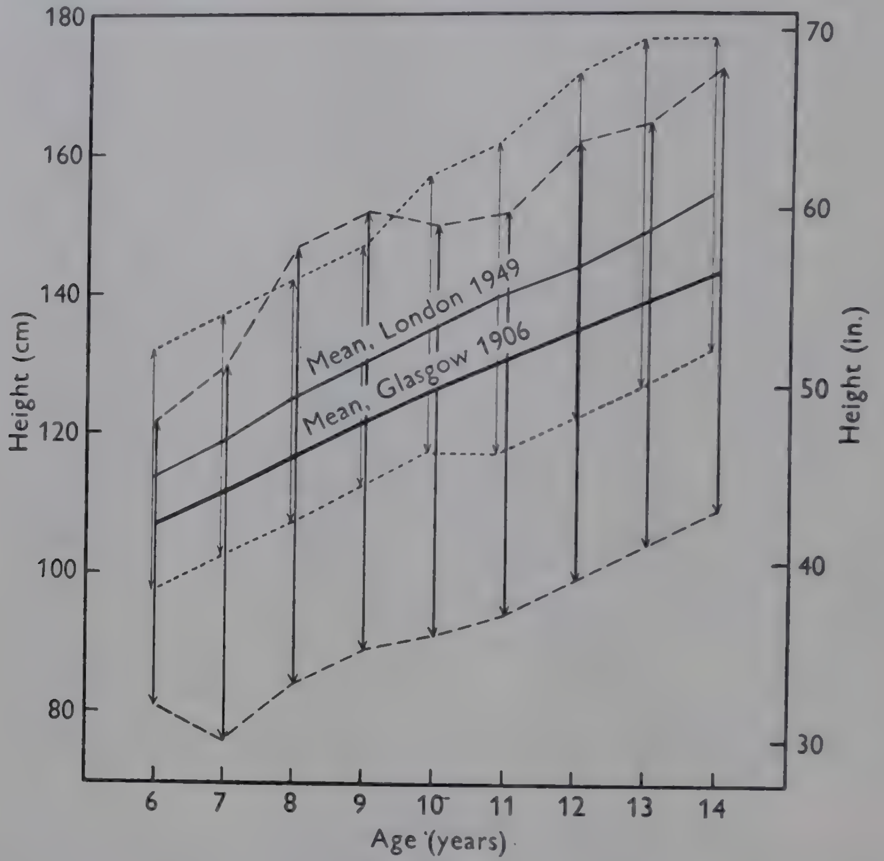

Fig. 8. Mean values and ranges for height of London boys in 1949 and Glasgow boys in 1906. Note the marked difference in the lower limit and little difference in the upper limit.

Secular change in relation to total variability of school population

So far only average values of height and weight have been considered. It is interesting to compare the secular change in growth with the variability of the school population at one time. The 1949 London report gives the standard deviations of both heights and weights. The secular change in London boys over a period of 40 years is almost identical with the standard deviation for weight and slightly in excess of it for height. The means and ranges of the heights of Glasgow boys in 1906 and London boys in 1949 are compared in Fig. 8. Despite the considerable difference between the mean figures, the 1949 London mean lags still further behind the Glasgow 1906 upper limit. The Glasgow range, which has been modified to allow for the fact that the Glasgow sample was larger than the London sample, is much larger than the London range. The Glasgow distribution is markedly skew, whereas the London one is fairly symmetrical. These facts suggest that the secular change reduces the scatter by relatively greater changes in those who are most retarded. This is confirmed by the relatively greater improvement in the Glasgow children from one-apartment houses compared with those better circumstanced.

Growth indices and body build

The growth-rate indices of height and weight provide a convenient way of studying body proportions or build both in individuals and in groups. If the two indices are equal then, whether growth is advanced or retarded, it is along the standard track and the build, so far as it is determined by height and weight, corresponds to the 1938 London standard. A weight index greater than the corresponding height index implies a stocky build and the converse indicates a slim build. The range of difference between the weight index and the height index of individuals is about ± 30, e.g. among boys aged 10 years of average height (height index 100) some weigh as much as an average boy of 13 years and some as little as an average boy of 7 years (weight indices 130 and 70 respectively).

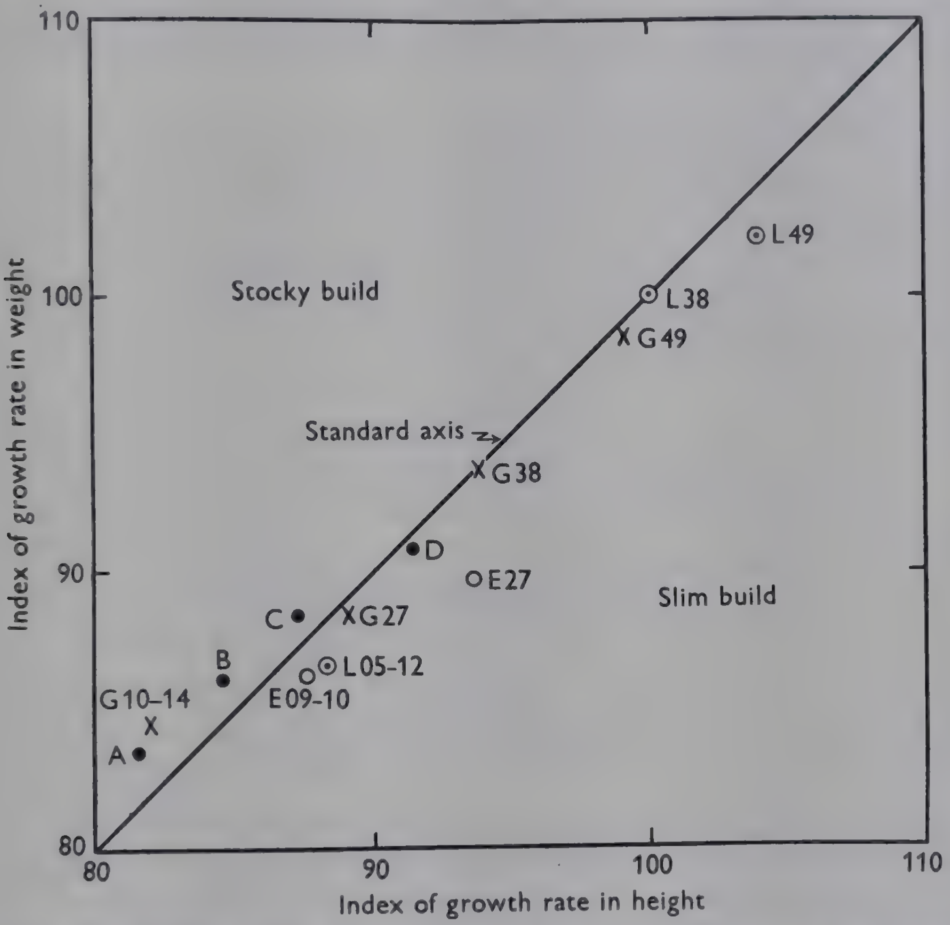

Fig. 9. Growth-rate indices of height and weight and the standard axis along which they are equal. The growth rate of London boys in 1938 is taken as the standard and is 100 on both scales. Deviations from the axis indicate a difference of body build. L 05–12, L 38, L 49 = London boys in 1905–12, 1938 and 1949; G 10–14, G 27, G 38, G 49 = Glasgow boys in 1910–14, 1927, 1938 and 1949; A, B, C, D, school groups in Glasgow in 1906; E 09–10, E 27, English boys in 1909–10 and 1927.

Fig. 9 shows the group growth-rate indices of height and weight for London, Glasgow and English schoolboys in relation to the standard axis. It demonstrates the marked advance during this century and also illustrates the fact that despite the marked changes in rate of growth (or of height and weight at a given age) the changes in build are extremely small in comparison with the range in the school population. This applies to all the age groups, not merely to the means. A rather fine point that may also be noticed is that with more rapid growth there is on the whole a tendency to a slimmer build, an oblique movement across the axis from left to right. The movement from London 1905–12 to London 1938 is an exception possibly due to sampling errors. It has been suggested that the oblique movement is due to a decreased incidence of rachitic deformity, but that is only a contributory cause. The figures for the London health divisions between 1938 and 1949 show that the north-east division crossed the line from left to right and the south-west division, which was already to the right in 1938, moved still further out.

In so far as the Glasgow 1906 groups are evidence of differences of nutrition they show that the dominant effect of undernutrition is retardation of growth and that an increased weight over that expected from age and height is not a reliable criterion of good nutrition. As to the cause of the secular change itself the only plausible explanation is a gradual improvement in nutrition.

DISCUSSION

To regard the secular change in growth merely as an increase of height and weight or even as a percentage increase in these measurements is misleading; e.g. Daley (1950) states that children of to-day are members of a generation altogether of greater physique. Now, neither the absolute nor the percentage increases in height are maintained into adult life. It is at present debatable whether or not there is any secular increase in the mean adult maximum height attained (Morant, 1948, 1950; Kemsley, 1950); but, if there is, it is certainly much less than the 3–4 in. of absolute increase or 6 % or so of percentage increase that has taken place in children during the past 40 years. On the other hand, to regard the change as an acceleration of the rate of growth is in agreement with the observed earlier attainment of maximum height; it does not preclude a secular increase in that height, and some increase seems likely with the elimination of retarded growth during childhood. The slow growers compensate to some extent by growing longer. This follows from the fact that the standard deviation of height reaches a maximum about the age of puberty and then declines. Growth and nutrition are intimately related and rate of growth is an index of nutrition. It may well be that the growth-rate indices will prove of value in assessing the nutrition of a group of schoolchildren, though there are too many other factors involved for them to be of much use with one individual.

SUMMARY

1. Published data of heights and weights of schoolboys in London and Glasgow during the present century have been examined and compared graphically and analytically.

2. In some 40 years the average height of London schoolboys has increased by about 7·5 cm (3 in.) and the average weight by about 4 kg (9 lb.). The Glasgow figures are similar.

3. The problem of comparing surveys of heights and weights is discussed.

4. Two units have been introduced, a year's growth in height and a year's growth in weight.

5. These lead to the concept of a growth age, and tables based on 1938 London averages are given for age corresponding to height and age corresponding to weight.

6. Growth ages in height and weight are proportional to chronological age.

7. The ratios give two indices of growth, growth rate in height and growth rate in weight.

8. Special methods are given for evaluating the growth indices of groups of children.

9. Secular changes in growth are similar to the differences in growth associated with differences in social conditions.

10. The secular change in 40 years approximated to one standard deviation of the measurements of the 1949 London boys.

11. The difference of the two indices is an index of body build.

12. The principal conclusion is that schoolchildren of to-day are growing faster than children of previous generations. As a result of the accelerated growth adult stature is earlier attained but not necessarily increased.

13. The indices of growth may be of value as indices of nutrition.

My thanks are due to Professor R. C. Garry for his interest in this work. I am also indebted to Dr I. D. Ferguson for his helpful criticism.

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The Nutrition of Nigerian Peasants, with Special Reference to the Effects of Deficiencies of the Vitamin B Complex, Vitamin A and Animal Protein

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(*Received* 21 *March* 1951)

Much of the present knowledge relating to the specific effects of deficiencies of single nutrients has been derived from relatively short-term experiments upon animals or human beings. The longer human experiments, e.g. those of Ancel Keys and his co-workers (Keys, Henschel, Taylor, Mickelsen & Brozek, 1945) into the results of deficiency of vitamins of the B complex, of Crandon, Lund & Dill (1940) into the effects of ascorbic-acid deficiency, or those of the Medical Research Council (Hume & Krebs, 1949) into the results of vitamin A deficiency, from 6 months to 1 or 2 years in duration, involve small numbers under conditions of diet and environment far removed from those to which the subjects are accustomed.

The confusion which may arise in assessing the effects of deficiencies of single nutrients has often been due to a failure to appreciate the importance of the interaction of one nutrient with another in the prevention of pathological processes. Examples are the interdependence of sulphur-containing amino-acids and tocopherol in the prevention of liver necrosis (Gyorgy, 1947; Himsworth & Lindan, 1949), and the interrelationship of vitamins A and C, about which conflicting views are expressed (Vedder & Rosenberg, 1938; Mayer & Krehl, 1948; Sharman, 1949). New knowledge of anti-vitamins and of intestinal synthesis of vitamins has greatly modified previous simple views of the development of deficiencies (Kodicek, 1948–9). In reviewing surveys of nutritional status carried out in the United Kingdom from the beginning of the second World War up to 1945, Marrack (1948–9) said that ‘we need base-lines from which to make comparisons’ and ‘we need to study groups of individuals, not only by making laboratory investigations, but also by finding out what they eat, how well they are, and what happens to them in the future’.

This nutritional study is the continuation of an attempt to record what Nigerian peasants eat, and to correlate their states of health and present diets. It is hoped that the data presented may be of value to others interested in the problems, both nutritional and administrative, which are associated with undernutrition in tropical communities.

METHODS AND RESULTS

Plan and scope of the investigation

The first part of the investigation (Nicol, 1949) was carried out in Northern Nigeria, on three groups of peasants whose staple foodstuffs were grains (*Sorghum vulgare* and *Penisetum typhoideum*). One of the groups obtained plentiful supplies of carotene from red palm oil, whereas the other two consumed very little of this nutrient. One group, as a result of a larger intake of sorghum, ingested more riboflavin than the others. These grain diets all contained ample amounts of aneurin and nicotinic acid, and provided adequate calories for normal physical exertion. From correlating differences in the diets with the clinical features presented by the different tribes, certain conclusions were drawn concerning the effects of deficiencies of vitamin A and riboflavin upon the physical status of these peoples.

The observations here recorded for the first time were made in Southern Nigeria among two tribes living in the Niger Delta, one being Ijaw fishermen and the other Urhobo farmers. As the staple foodstuffs of these tribes are starchy roots and fish it was believed that the intake of B-complex vitamins, including aneurin and nicotinic acid, would be low and that comparison of the clinical features presented by the southern tribes with those recorded in the northern areas would give some indication of the results of specific deficiencies of aneurin and nicotinic acid. Further, it was considered that information of value might be obtained from the association, in the diets of the fishermen, of a high intake of animal protein with a low intake of B-complex vitamins. For comparison a group of wealthy southern Nigerian traders, mixed Urhobo and Ijaw, was similarly investigated. The diets eaten by these traders are more liberal and varied than those of the peasants.

The clinical and dietary survey was carried out in 1949 and 1950 in Warri Province. The Ijaw fishermen live in Soragbemi, a village situated on one of the creeks of the Niger Delta, in mangrove swamp, 5° 30′ N., 5° 30′ E. The Urhobo farmers live in Illu, a village on the borders of the freshwater swamp and high forest zones, 5° 40′ N., 6° 10′ E. The traders live in the comparatively civilized township of Warri, 5° 30′ N., 6° 0′ E. The climate is hot and moist throughout the year, with an annual rainfall of 120–180 in. The whole district is only a few feet above sea-level.

The tribes examined in 1947 and 1948 (Nicol, 1949) live in villages in the zones of guinea- and sudan-savannah, between 9° and 12° N., and 500–1400 ft. above sea-level, in a much drier climate with a mean annual rainfall of 40–60 in.

Thus the whole investigation provides information on the diets and the clinical features of five groups of peasants and of one group of wealthy traders as follows:

Region	District	Occupation
Northern Nigeria	Bida	Farmers
	Kontagora	Farmers
	Zuru	Farmers
Southern Nigeria	Soragbemi	Fishermen
	Illu	Farmers
	Warri	Traders

Dietary studies

Southern families whose diets were investigated

Three typical families in each area were selected for investigation. All food eaten by the adults of the compounds was weighed and measured for three periods of 7 days throughout the year April 1949 to April 1950, the periods being selected in accordance with the seasonal availability of staple foodstuffs. Only individuals considered to be 12 years of age or over were included in the investigation, the numbers in the families in each area being as follows: Soragbemi, ten males, thirteen females; Illu, nine males, twelve females; Warri, five males, eight females. All weighing and recording was carried out, under supervision, by a hospital attendant, specially trained for the purpose, with a practical knowledge of local conditions. A balance weighing to $\frac{1}{8}$ oz. was used; the conversion factor to grams was 28·35.

Estimation of intake of food and nutrients

All foods were weighed in bulk before cooking, and individual helpings were weighed before eating, the amount of each foodstuff, as uncooked weight, being calculated by proportion. When food was eaten from a communal dish, as was frequently the case with dry cassava flour and yams, the amount consumed *per caput* was calculated by dividing the total quantity eaten by the number of consumers. Food eaten outside the compounds was weighed on the spot, or was estimated by asking the farmer or fisherman to bring back an equal quantity to the compound on his return. The amounts consumed by visitors to the various families were allowed for by this method of calculating individual intakes at each meal. The mean daily intake *per caput* for each group throughout the year was calculated from the figures for each period of investigation and from these data the nutrient and energy values were determined by using Platt's (1945) tables of representative values, additional values being taken from Nicholls (1945), McLester (1949), and from the tables of the Food and Agriculture Organization of the United Nations (Chatfield, 1949).

Estimations of the edible portions, percentage waste and moisture content of certain foodstuffs, e.g. monkey meat, porcupine and some of the larger fishes, were made in the Warri Hospital laboratory. The alcohol content of locally distilled spirits was estimated by analysis of samples from the villages, and the mean value was found to be 60 %. I am indebted to the Laboratory Service for these figures.

Consideration of the diets eaten

The staple foodstuffs of the farmers and fishermen in the southern areas are cassava and yam. The fishermen eat large amounts of fish, but the farmers eat smaller quantities of fish and supplement their protein ration with meat from small land animals, porcupine, pangolin, monkey and snails. Yams are more important than cassava in the diet of the farmers, the converse being the case with the fishermen. Pulses are used in small quantities by the farmers. Apart from peppers, fresh vegetables are seldom cultivated by the peasants in Warri Province, and wild-growing herbs rarely used. Palm oil is plentiful and is the only cooking fat. Fruit is seldom, if ever, eaten.

Palm wine, tapped from the red-oil palm (*Elaeis guineensis* Jacq.) is the main beverage used by the farmers; the fishermen drink large amounts of 'illicit gin', distilled from palm wine, sweet potatoes and plantains.

The foodstuffs eaten by the farmers, fishermen and traders in the southern region during 1949–50 are shown in Table 1. For details of the foodstuffs of the tribes in the northern region in 1947–8 see Nicol (1949).

Table 1. *Average daily food consumption of Southern Nigerian peasant farmers, fishermen and traders, based on a survey of adults in Warri Province,* 1949 *and* 1950

Foodstuff	Scientific name	Warri traders (g)	Illu farmers (g)	Soragbemi fishermen (g)
Wheat flour (70%)	*Triticum vulgare* Host Cultigen	70	—	—
Rice, milled	*Oryza sativa* Linn.	76	—	—
Maize: mature yellow	*Zea mays* Linn.	30	29	15
immature		10	7	0
Cassava: flour	*Manihot utillissima* Pohl.	109	143	186
starch		102	172	92
'fufu'		—	63	—
fresh tuber		—	—	77
Yam	*Dioscores sativa* Linn.	300	633	63
Cocoyam	*Colocasia antiquorum* Schott.	—	—	12
Plantain, green	*Musa paradisiaca* Linn.	17	14	13
Banana	*M. sapientium* Linn.	22	—	—
Sweet potato	*Ipomoea batatas* Linn.	—	—	19
Cow pea	*Vigna sinensis*	15	13	—
Jak fruit	*Artocarpus integer* Merr.	—	3	—
Groundnuts	*Arachis hypogaea* Linn.	4	2	—
Pumpkin seed	*Cucurbita* spp.	3	1	—
Coconut kernel	*Cocos nucifera* Linn.	2	—	—
Oil-bean kernel	*Pentaclethra macrophylla* Bth.	—	1	—
Dika-nut kernel	*Irvingia gabonensis* Baill.	1	—	—
Kola-nut kernel	*Kola acuminata* Schott. and Endl.	3	1	1
Onions	*Allium cepa* Linn.	4	—	—
Okra, fresh	*Hibiscus esculentus* Linn.	6	—	—
Green leaves from bush	*Corchorus* spp., *amaranthus* spp. and others	12	—	—
Peppers, red: dry	*Capsicum frutescens* Bl.	3	3	4
fresh		2	3	1
green, fresh		5	2	1
Tomato, fresh	*Lycopersicum esculentum* Mill.	4	2	—
Avocado pear	*Persea americana* Mill.	26	—	—
Guava	*Psidium guajava* Linn.	4	—	—
Mango	*Mangifera indica* Linn.	20	2	—
Pawpaw	*Carica papaya* Linn.	12	—	—
Orange	*Citrus sinensis* sp.	10	—	—
Custard apple	*Annona reticulata* Linn.	7	—	—
Beef	—	96	—	—
Mutton	—	12	—	—
Pork, fat	—	16	—	—
Liver	—	4	—	—
Eggs	—	10	—	—
Fish*: fresh	—	62	18	165
dried	—	28	18	78
Shrimps, dry	—	2	—	1
Prawns, fresh	—	—	—	4
Oysters, local	—	—	—	7
Goat	—	—	6	—
Monkey, dried	Various species	—	17	—
African snail	*Archachatina* sp.	3	3	—
Palm weevils	*Rhynchophorus phoenicis*	1	1	—
Frogs' legs	*Rana mascarenensis*	—	3	—
Pangolin	*Manis longicaudata* and *M. tricuspis*	—	3	—
Porcupine	*Aterurus africanus*	—	2	—
Cane rat	*Thryonomys swinderianus*	—	1	—
Giant Gambian rat	*Cricetomys gambianus*	—	2	—

* Mixed lake and estuary.

Table 1 (*cont.*)

Foodstuff	Scientific name	Warri traders (g)	Illu farmers (g)	Soragbemi fishermen (g)
Sardines, tinned	—	3	—	—
Milk: sweetened condensed	—	5	—	—
evaporated	—	28	—	—
Palm oil	From *Elaeis guineensis* Jacq.	36	35	27
Margarine	—	5	—	—
Sugar-cane, stem	*Saccharum officinarum* Linn.	26	3	4
Jams, imported	—	8	—	—
Palm wine (ml.)	From *Elaeis guineensis* and *Raphia vinifera*	120	250	103
'Illicit gin' (ml.)	—	20	13	107
Gin, European (ml.)	—	26	—	—
Whisky (ml.)	—	23	—	—
Brandy (ml.)	—	15	—	—
Beer, imported (ml.)	—	100	—	—
Tea	—	4	—	—
Coffee	—	3	—	—
Nut (Urhobo-'ewoie')	—	—	1	1
Salt: imported	—	10	5	7
mangrove	—	4	3	4

Preparation and cooking of foodstuffs

Two main meals, morning and evening, are eaten by the farmers and fishermen. The traders tend to conform to the three-meal pattern of the United Kingdom, with a snack or tea in the morning and afternoon.

The methods of preparing cassava in the southern areas are as follows. 'Garri', or flour, is made by sieving freshly peeled tubers through fine-meshed, home-made sieves, and drying the resulting pulp in wide basins over charcoal or wood fires. The flour contains very little moisture, and the vitamin content is low. It is boiled with a little water and made into a paste or gruel. 'Farina', another preparation of flour, is made by sieving fresh cassava tubers and wrapping the pulp in wicker baskets or leaves and then pressing these bags between heavy stones for 2 days to express the moisture. Then the pulp is teased out and sun-dried for 8–10 h. The resulting cake, hard and friable, is stored in sacks. It is eaten either dry or moistened, or used in place of 'garri' to make gruel. 'Starch' is prepared by heating pounded cassava with water in an open pot, and squeezing the dough through a cloth or fine wicker-work bag, the filtrate being heated in a pan until the starch sets. It is then wrapped in plantain leaves, and is eaten either cold or heated, or made into a paste with hot water. 'Fufu' is made by soaking whole tubers in a stream or pond for 2 or 3 days, peeling the rotting root and then sieving or pounding into a paste, which is boiled with a little water before eating.

Yams are prepared for cooking by peeling and slicing or pounding, and are eaten after boiling for about an hour. Sometimes yams are fried in palm oil or roasted in wood ashes.

The soups used by both farmers and fishermen have little variety, consisting almost invariably of peppers, palm oil, meat or fish and salt, either imported or made from mangrove or other wood ash. Edible earths are not used in this province.

Palm fruit is used in two ways. In one the fruit is boiled in water for about 20 min and kneaded to remove the pulp from the kernel. This watery pulp, containing oil and the fibrous stroma of the fruit, is the basis of most soups. In the second the watery pulp is simmered and skimmed; the oil so obtained is used for frying fish or yams and bottled for sale in the local markets. Many estimations in the villages showed that these methods yield about 1½ oz. oil from 1 lb. palm fruit, the weight of kernels remaining being 11–12 oz. In calculating the nutrient value of palm oil as used in the diets, it was taken that 1 lb. fruit yields 1½ oz. oil.

Fish, dried or fresh, is usually boiled, but occasionally fried or roasted. It is seldom eaten raw.

The fishermen often mix ground peppers with the grated kernel of a nut (Urhobo-'ewoie') which has not been identified botanically. The farmers allow the kernel of the oil bean tree to ferment before making it into a cake or savoury flavouring for soups. The amounts of these two foodstuffs used by the peasants are small, and it is not thought that lack of knowledge of their nutrient composition makes an appreciable difference to the calculated nutrient values of the diets.

Nutrient values of the peasants' diets

The analysis of the diets, including those of the northern tribes, is shown in Table 2. Allowance has not been made for destruction of nutrients by cooking. It is probable that some aneurin is lost, as fish and yams, the foods providing most, are usually boiled and the water discarded. As fish is generally boiled before eating it is unlikely that destruction of aneurin by enzymes derived from the fish itself is an important source of loss (Melnick, Hochberg & Oser, 1945). If an allowance of 67 % is made for destruction of ascorbic acid in cooking, each of the diets still provides 10 mg or more daily, the amount found by the Medical Research Council (1948) to prevent clinical scurvy, but the Bida farmers alone of the peasants obtain more than the 20 mg recommended by the British Medical Association Committee on Nutrition (1950) as a daily allowance for moderately active adults, and only they reach the figure of 30 mg suggested by Platt (1946) and the Medical Research Council (1948) to be necessary to cover all requirements for active life and inherent individual variability.

Comparisons of the nutritive values of the diets of the six groups has been made with the daily 'allowances' recommended by the (U.S.A.) National Research Council (1948). In these, the mean adult weights of each group have been taken into account, and a reduction of 12½ % has been made from the number of calories recommended by the Council to allow for the reduction of basal and total metabolic rates found in tropical climates (Keys, 1949). The data have been expressed as Cal./lb. body-weight (Table 3). It was impossible to estimate the energy expenditure of the peasants, but at certain times they are very active, the northern farmers in the fields and when boxing and wrestling, the fishermen when paddling long distances often against strong tides and currents. It seems justifiable to classify all groups as 'moderately active'. Protein intake has been expressed as g/lb. body-weight (Table 3); aneurin, riboflavin

Table 2. *Calculated daily nutrient intakes of Nigerian peasants and traders in six areas*

		Protein										
Group and area	Cal.	Animal (g)	Vegetable (g)	Fat (g)	Carbo-hydrate (g)	Vitamin A* (i.u.)	Aneurin (mg)	Riboflavin† (mg)	Nicotinic acid (mg)	Ascorbic acid‡ (mg)	Calcium (mg)	Iron (mg)
Soragbemi fishermen	2191	68	12	39	379	5655	0·30	0·68	12·1	40	2894	24
Illu farmers	2252	17	26	43	424	7348	0·69	0·65	8·9	30	664	21
Bida farmers	2639	5	70	44	486	5174	3·20	0·76	19·0	98	489	40
Kontagora farmers	2431	1	72	26	476	625	3·20	0·76	21·0	44	344	42
Zuru farmers	2947	2	87	24	595	713	3·70	0·98	25·0	34	581	57
Warri traders	3003	50	34	86	473	9206	1·02	1·37	18·1	71	1302	21

	Contribution to total calories (%)			Composition of diets (% by weight)		
	Protein	Fat	Carbohydrate	Protein	Fat	Carbohydrate
Soragbemi fishermen	14·6	16·4	69·0	16·1	7·8	76·1
Illu farmers	7·7	17·0	75·3	8·4	8·4	83·3
Bida farmers	11·3	15·1	73·6	12·3	7·3	80·4
Kontagora farmers	12·1	9·6	78·3	12·8	4·5	82·7
Zuru farmers	12·1	7·2	80·7	12·5	3·3	84·2
Warri traders	11·2	25·8	63·0	13·1	13·4	73·5

* 1 i.u. of preformed vitamin A taken as equivalent to 3 i.u. of vitamin A in the form of carotene (Platt, 1945).

† The differences in riboflavin intake between Zuru and the other areas are significant (Zuru-Bida 0·220 ± 0·0413; Zuru-Kontagora 0·221 ± 0·0425; Zuru-Soragbemi 0·300 ± 0·0512; Zuru-Illu 0·328 ± 0·0520).

‡ No allowance made for losses of ascorbic acid in cooking.

and nicotinic acid as mg/lb. body-weight and mg/1000 Cal., and aneurin also as the ratio aneurin/non-fat Cal. (Passmore, 1948) (Table 4).

From these data, none of the groups appears deficient in calories for 'moderate activity' per lb. body-weight. The Illu farmers are the only group deficient in total protein, but the supply of animal protein to the northern farmers is negligible. The fat content of the peasants' diets, even in the areas where palm oil is plentiful, is less than a third of the amount normally found in good European diets.

Table 3. *Calculated amounts of protein and calories supplied by the diets per lb. body-weight, compared with (U.S.A.) National Research Council recommendations for moderately active men and women of mean weight* 139 *lb.,* 12½ % *reduction in calories being allowed for the effect of climate upon basal and total basal metabolic rate (Keys,* 1949)

	Mean weight, male and female (lb.)	Cal.	Total protein (g)	Animal protein (g)	Vegetable protein (g)
Soragbemi fishermen	119	18·4	0·67	0·57	0·10
Illu farmers	111	22·9	0·39	0·15	0·24
Bida farmers	116	22·8	0·65	0·04	0·61
Kontagora farmers	116	20·6	0·62	0·01	0·61
Zuru farmers	117	24·9	0·76	0·02	0·74
Warri traders	136	22·1	0·62	0·37	0·25
N.R.C. allowances*	139	17·0	0·47	0·31	0·16

* National Research Council (1948).

Calcium is 'deficient' in all peasant diets except that of the fishermen. Iron is present in amounts considered adequate by European standards, but these do not allow for the heavy intestinal infestations and endemic malaria encountered among African peasant populations.

Important differences in vitamin intakes have been noted between one area and another. First, carotene is plentiful in the diets in Bida, Soragbemi and Illu, but scarce in Kontagora and Zuru, owing to the geographical distribution of the red-oil palm. Secondly, the intake of aneurin is very low in Soragbemi and Illu but plentiful in Bida, Kontagora and Zuru, because the staples in the first two areas are starchy roots and in the other three areas sorghum and millet. Thirdly, the intake of nicotinic acid in Soragbemi and Illu is much lower than elsewhere, although that in Soragbemi is 'adequate' judged by National Research Council (1948) standards. Fourthly, the intake of riboflavin is low in all areas, but that in Zuru is 25–35 % higher than in the other areas. This is due to the large quantities of sorghum eaten by the Zuru farmers.

Clinical studies

Clinical examination

A clinical examination of all the individuals whose diets were measured, and of others of the same economic status, was carried out in each area. Since Sinclair (1948) doubts the ability of one observer to record clinical signs consistently, great care was

Table 4. *Calculated amounts of aneurin, riboflavin and nicotinic acid in the diets of Nigerian peasants and traders from six areas, expressed as mg/lb. body-weight and as mg/1000 Cal. in each diet*

	Aneurin		Riboflavin		Nicotinic acid		Ratio aneurin : non-fat Cal.
	(mg/lb. body-weight)	(mg/1000 Cal.)	(mg./lb. body-weight)	(mg/1000 Cal.)	(mg/lb. body-weight)	(mg/1000 Cal.)	
Soragbemi fishermen	0·002	0·14	0·006	0·310	0·10	5·5	0·20
Illu farmers	0·006	0·31	0·006	0·288	0·08	4·0	0·37
Bida farmers	0·028	1·25	0·006	0·288	0·16	7·2	1·43
Kontagora farmers	0·028	1·32	0·006	0·317	0·18	8·6	1·46
Zuru farmers	0·031	1·25	0·008	0·332	0·21	8·5	1·35
Warri traders	0·008	0·34	0·011	0·456	0·13	6·0	0·46
N.R.C. allowances*	0·010	0·50	0·012	0·611	0·09	5·0	—

* National Research Council (1948).

taken to standardize records by constant reference to photographs previously taken. The physical and mental development of the African who assisted at all examinations was also a useful standard reference of health.

The clinical criteria used in the examination are stated in Table 5. Particular care was taken in the examination of the liver, and in questioning each individual about any previous history of jaundice or haematuria. Haemoglobin was estimated by Sahli's method and the instrument was checked against a Haldane haemoglobinometer (100 % (Haldane) = 14·8 g Hb/100 ml.) in the United Kingdom at the end of the investigation.

All mothers were questioned regarding the numbers of children alive, stillbirths and miscarriages, and this information was cross-checked with the husbands and neighbours (Table 6).

Microscopic examination of the urine and faeces of samples of each population was carried out in the villages, and the weights of large samples were recorded, using an accurate yard-arm machine.

Clinical findings

The sex and age incidence of the samples of the population are given in Table 7. The incidence of clinical abnormalities and the criteria on which they were assessed, are given in Table 5.

The general appearance of individuals in each sample, the mean haemoglobin levels and the incidence of hepatic disease and of symptoms associated with the aetiology of hepatitis and cirrhosis of the liver are presented in Table 8. The mean weights and sizes of the samples taken are given in Table 9, and the infestation rates and splenic indices in Table 10.

The general appearance of individuals, who were classified into three groups, good, fair, and poor according to mental and physical development and alertness, muscular and skeletal development and any other features disclosed by examination, was found to be best in the Warri traders, the Soragbemi fishermen being a close second, and the Illu and Zuru farmers worst. A significant difference in sex incidence for any of the features considered was not found, but staring hair occurred most often below the age of 10 years, and the incidence of pigmentation of the sclera, thickening of the conjunctiva and gingivitis increased with age.

When reporting the nutritional status of the northern tribes the clinical features were grouped into those found most frequently in areas where deficiency of a particular nutrient occurred, or where the deficiency was of greater degree than elsewhere (Nicol, 1949). In this report the same method has been applied and extended to include the southern populations, with the object, first, of confirming or otherwise the conclusions previously drawn concerning the effects of deficiencies of vitamin A and riboflavin and, secondly, of comparing the incidence of features found in areas where the supply of aneurin and nicotinic acid is low with that occurring in areas where these nutrients are plentiful. This grouping of the clinical features is given in Table 11.

Table 5. *Clinical features: classification and incidence in five groups of Nigerian peasants and in one group of Nigerian traders*

Clinical features	Grade	Criteria upon which features assessed	Warri traders (64)* (%)	Soragbemi fishermen (80)* (%)	Illu farmers (80)* (%)	Bida farmers (92)* (%)	Kontagora farmers (93)* (%)	Zuru farmers (79)* (%)
A. Skin								
Generalized xerosis	1	General dryness of skin without scaling	0	10	13	20	44	41
	2	Dry scaly skin with accentuated pattern	0	1	2	0	46	47
	3	As in grade 2, but with follicular hyperkeratosis	0	3	1	0	6	8
'Elephant skin'	1	Thickening over elbows and knees only	10	58	63	50	38	22
	2	Thickening includes dorsum of feet and ankles	2	1	10	8	50	60
	3	Marked thickening over feet and ankles	0	0	0	0	6	6
Folliculosis	1	A few prominent follicles on forearms and thighs	24	49	54	55	53	56
	2	Numerous follicles on arms, thighs and buttocks	12	14	3	12	11	14
Nasolabial seborrhoea	1	Accumulations of sebaceous matter at base of alae nasae	12	43	56	47	50	34
	2	As in grade 1, but involving chin, cheeks, forehead	2	5	6	28	15	9
Permanent gooseflesh	—	Areas on neck or trunk where all follicles show cutis anserina	6	29	25	24	22	14
'Crackled' skin	—	Skin of legs dry, scaly and accentuated pattern	6	12	18	9	32	43
Atrophic skin	—	Skin of legs shiny, smooth and hairless	20	63	79	71	71	75
Ulcers and scars	—	Active ulcers or scars on feet or ankles	18	39	46	50	44	49
Staring hair	—	Dull, straight or lax hair, with or without depigmentation	2	12	9	12	44	50
B. Eyes								
Appearance of bulbar conjunctiva and sclera	1	Pigmentation of sclera in palpebral fissure	37	33	44	32	44	35
	2	Thickening and wrinkling without dryness	16	48	38	39	34	30
Corneal opacities	1	Dryness of conjunctiva with early corneal haze	0	0	1	2	4	10
	2	Frank xerophthalmia with or without ulcer	0	0	1	0	1	3
	3	Superficial corneal opacity without dryness of conjunctiva	2	10	9	2	10	5
Night-blindness	—	Present according to subject's own statement	0	0	0	3	32	42
Photophobia	—	Clinical observation of patient's complaint	0	10	13	9	8	11
Cataract	—	Opacity of lens visible to naked eye	1	3	6	3	4	4
Vascularity of bulbar conjunctiva	1	Abnormal injection of minor degree	27	50	61	56	43	33
	2	Marked redness and injection	2	3	6	5	9	10

Table 5 (*cont.*)

Clinical features	Grade	Criteria upon which features assessed	Warri traders (64)* (%)	Soragbemi fishermen (80)* (%)	Illu farmers (80)* (%)	Bida farmers (92)* (%)	Kontagora farmers (93)* (%)	Zuru farmers (79)* (%)
C. Lips and tongue								
Angular stomatitis	1	Slight maceration or scarring	3	50	50	47	55	30
	2	Marked maceration or fissuring	1	3	6	8	3	6
Colour changes in tongue	1	Reddened and sore-looking	2	23	31	36	42	39
	2	Magenta or lilac	2	40	38	13	12	11
Atrophy of epithelium of tongue	1	Marginal atrophy only	3	39	33	34	31	30
	2	Patchy atrophy over surface of tongue	2	7	14	15	5	7
	3	Bald and smooth	0	1	0	2	2	0
'Pebbling' of filiform papillae	—	Enlargement and prominence of these papillae	4	33	48	21	30	20
Fissuring of tongue	1	Longitudinal only	17	24	20	24	26	25
	2	Longitudinal and transverse, and inflamed	1	3	5	5	9	13
Indentations	—	Oedematous indentations of margins	4	24	33	40	20	24
D. Other signs								
Calf tenderness	—	Tenderness elicited by pressure on calf by hand	18	40	31	3	2	3
Gingivitis	—	Infective condition of gums	29	33	45	70	59	51
Dental caries, malocclusion and missing teeth	—	Caries seen easily in one or more teeth	12	15	35	36	29	28
Rachitic signs	—	Two or more clinical signs of this disease recorded in each case	0	8	13	2	1	1

* No. of people examined.

Table 6. *Child mortality and failed pregnancies in five areas in Nigeria*

	No. of mothers	No. of children alive	Children dying between birth and 1 year	5 years	over 5 years	Infant-mortality rate
Soragbemi fishermen, 1949–50	44	100	68	12	16	347
Illu farmers, 1949–50	36	54	54	12	6	429
Bida farmers, 1947–8	41	72	30	7	9	254
Kontagora farmers, 1947–8	30	87	55	8	7	351
Zuru farmers, 1947–8	26	36	32	9	7	276

Abortions, miscarriages and stillbirths: Soragbemi, 12; Illu, 12; Bida, 24; Kontagora, 14; Zuru, 16.

Table 7. *Age and sex incidence of samples of the population examined clinically in six areas of Nigeria*

	0–10 years		11–20 years		21–30 years		31–40 years		41–50 years		Over 50 years		
District	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	Total
Soragbemi	5	6	8	8	10	8	10	6	6	4	5	4	80
Illu	4	3	10	9	9	11	9	8	6	5	3	3	80
Bida	4	4	11	9	11	17	8	9	6	6	4	3	92
Kontagora	8	9	17	14	6	8	6	11	2	5	4	3	93
Zuru	6	7	13	7	8	7	6	8	6	4	4	3	79
Warri	2	3	6	4	6	5	6	10	7	8	4	3	64
											Total		488

M. male; F. female.

Table 8. *General appearance, incidence of disorders of the liver, mean haemoglobin levels, of five groups of Nigerian peasants and one group of Nigerian traders*

	Warri traders (64)* (%)	Soragbemi fishermen (80)* (%)	Illu farmers (80)* (%)	Bida farmers (92)* (%)	Kontagora farmers (93)* (%)	Zuru farmers (79)* (%)
General appearance: good	70	67	38	47	43	38
fair	26	30	56	47	48	46
poor	4	3	6	6	9	16
Clinically demonstrable liver disease	3	8	19	15	12	9
Previous history of jaundice	3	8	6	5	6	6
Previous history of haematuria	1	1	0	9	3	6
Mean Hb readings	82	76	70	70	73	76
Standard deviation	11·80	11·80	10·60	10·62	10·90	11·95
Standard error of mean	1·48	1·32	1·19	1·13	1·11	1·36

* No. of people examined.

Table 9. *Mean weights of five samples of the peasant population of Nigeria, and of one sample of wealthy native traders*

	Warri traders (484)*	Soragbemi fishermen (757)*	Illu farmers (1085)*	Bida farmers (638)*	Kontagora farmers (702)*	Zuru farmers (685)*
Mean weight (lb.)	136	119	111	116	116	117
Standard deviation	22·90	12·65	15·85	13·64	12·80	11·98
Standard error of mean	1·04	0·46	0·48	0·53	0·48	0·46

* Number in sample, ages 16–65 years.

Table 10. *Percentage incidence of parasitic infestations in six areas of Nigeria*

	Warri traders (100)*	Soragbemi fishermen (150)*	Illu farmers (150)*	Bida farmers (200)*	Kontagora farmers (200)*	Zuru farmers (200)*
Ankylostomiasis	73	96	95	95	57	65
Ascariasis	17	20	31	0	6	5
S. mansoni	0	0	1	0	5	4
S. haematobium	1	0	1	50	70	39
Trypanosomiasis	0	0	0	15	0	0
Dracontiasis	0	0	0	Infrequent	Frequent	Frequent
Splenic index 2–10 years, 100 children in each area	17	17	22	20	15	17

(I am indebted to Dr O. D. Macnamara for his figures concerning the infestation rates in Bida, Kontagora and Zuru, reported in 1945–6.)

* No. of people examined.

DISCUSSION

The incidence of hepatic disease and anaemia, which is almost always hypochromic and microcytic in type, was high, but the major nutritional diseases, beri-beri, pellagra and scurvy have not been encountered. However, the minor stigmata associated with nutritional deficiencies have been recorded very often.

The high rate of intestinal infestations (Table 10), particularly with hookworms, round-worms and schistosomes, is considered to be a more important factor in the production of anaemia than dietary deficiencies. Distinct from the loss of blood caused by these parasites is the amount of nutriment of which they deprive the host. Chance & Dirnhuber (1949) have shown that nematodes, trematodes and cestodes all contain large amounts of B-complex vitamins. For instance, analysis of *Ascaris lumbricoides* revealed 13·5 μg riboflavin, 10 μg aneurin and 200 μg nicotinic acid per 100 g dry weight of whole worm. They believe that the parasites absorb large quantities of these vitamins from the gut lumen. After a vermifuge, an African may pass some hundreds of ascaris and the effect of these upon the supplies of B-complex vitamins, whether ingested or synthesized in the intestine, must be considerable.

The numbers of peasants suffering from schistosomiasis was much lower in the south than in the north, and it was expected, as a result, that the incidence of cirrhosis of the liver would be less in this region. This has not been found, and it appears probable that nutritional factors are the chief cause of the high incidence of this disease in all areas.

Specific vitamin deficiencies

The clinical data, grouped as described on p. 43 and presented in Table 11, confirm the earlier conclusions that vitamin A deficiency results in dryness and depigmentation of the hair, general xerosis, follicular hyperkeratosis, 'elephant skin', 'crackled' skin and night-blindness. Corneal opacity is considered below. Many individuals showing all grades of xerosis, 'elephant skin' and 'crackled' skin have been found among those tribes whose diets contain ample quantities of carotene. It is believed that, in certain cases, these signs may result from a deficiency of ascorbic acid, and in others from an inability to utilize vitamin A as a result of disturbed liver

Table 11. *Grouping, according to nutrient intakes, of the clinical features observed in peasants from five areas of Nigeria*

	Soragbemi fishermen (80)*	Illu farmers (80)*	Bida farmers (92)*	Kontagora farmers (93)*	Zuru farmers (79)*
Mean intake of:					
Vitamin A (i.u.)	5655	7348	5174	624	713
Aneurin (mg)	0·30	0·69	3·20	3·20	3·70
Riboflavin (mg)	0·68	0·65	0·76	0·76	0·98
Nicotinic acid (mg)	12·1	8·9	19·0	21·0	25·0
Ascorbic acid (mg)†	26·7	10·0	32·7	14·7	11·3
Calcium (mg)	2894	664	489	344	581
Protein (g): animal plus vegetable = total	68 + 12 = 80	17 + 26 = 43	5 + 70 = 75	1 + 72 = 73	2 + 87 = 89

Clinical features (percentage incidence)

Group 1: roughly same incidence in each area

	Soragbemi fishermen	Illu farmers	Bida farmers	Kontagora farmers	Zuru farmers
Folliculosis	63	57	67	64	70
Atrophic skin	63	79	71	71	75
Ulcers and scars of ulcers	39	46	50	44	49
Thickened conjunctiva	48	38	39	34	30
Photophobia	13	10	9	8	11
Fissuring of tongue	27	25	29	35	38
Oedema of tongue	24	33	40	20	24
Corneal opacity, grade 3	10	9	2	10	5

Group 2: more frequent in Kontagora and Zuru than elsewhere

	Kontagora and Zuru	Soragbemi, Illu and Bida
Hair dry and staring	47	11
'Crackled' skin	37	13
Xerosis, all grades	32	5
'Elephant skin', grades 2 and 3	61	6
Corneal opacity, grade 1	7	1
Night-blindness	37	1

Group 3: less frequent in Zuru than elsewhere

	Soragbemi, Illu, Bida and Kontagora	Zuru
Nasolabial seborrhoea	63	43
Permanent gooseflesh	25	14
Injection of conjunctiva	60	48
Angular stomatitis	56	36

Group 4: more common in Soragbemi and Illu than elsewhere

	Soragbemi and Illu	Kontagora, Bida and Zuru
Calf tenderness	36	3
Magenta and lilac tongues	39	12
Signs of rickets	11	1

Group 5: more common in Illu than elsewhere

	Illu	Soragbemi, Kontagora Bida and Zuru
Enlargement of filiform papillae of tongue	48	26

Group 6: Less common in Soragbemi than elsewhere

	Illu, Bida, Zuru and Kontagora	Soragbemi
Red, sore-looking tongues	37	23
Dental caries	32	15

Significance: $P = 0{\cdot}05$ for each of the clinical features considered in groups 2–6.

* No. of people examined.

† 67% allowance made for destruction of ascorbic acid in cooking.

function or lack of fat to aid absorption of carotene. Analysis of those cases with severe clinical disorders of the liver showed that a high percentage presented the skin signs of vitamin A deficiency, corneal opacity and dryness of the conjunctiva, despite an adequate intake of carotene.

The figures also confirm the conclusions that riboflavin deficiency leads to the occurrence of angular stomatitis, nasolabial seborrhoea, increased vascularity of the conjunctiva and permanent gooseflesh. Lesions of the cornea resulting from riboflavin deficiency are considered below.

In Soragbemi and Illu it was found that magenta and lilac coloration of the tongue, tenderness of calves and rickets were more frequent than in the other three villages. It is considered justifiable to attribute the tenderness of calves to deficiency of aneurin, but why the other signs fall into this group is obscure.

'Pebbling' of the lingual papillae was observed more frequently in Illu than elsewhere. The intake of nicotinic acid in this area was only two-thirds of that recommended by the (U.S.A.) National Research Council (1948) as a daily allowance. This finding is in accordance with the views of Adamson & Platt (quoted by Abbott, 1950) that hypertrophy of the mucous membrane of the tongue is an early result of chronic deficiency of nicotinic acid.

Apart from the observations made above that a low intake of ascorbic acid may result in xerosis, follicular hyperkeratosis and 'crackled' skin in some cases, other signs of vitamin C deficiency were not encountered.

With regard to those signs that were found to occur with equal frequency in all areas (group 1, Table 11) it is possible that the long-continued ingestion of sub-optimal amounts of riboflavin leads to their appearance. Consideration of the clinical features presented by the Warri traders, however, which are discussed below, suggests that some of these signs are not nutritional in origin, but are the result of minor physical trauma.

Corneal opacities

From a consideration of the literature cited elsewhere (Nicol 1949) it would appear that corneal opacity due to vitamin A deficiency is usually associated with thickening and dryness of the conjunctiva, whereas the superficial keratitis which is held to result from lack of riboflavin is not so associated. The data now available from this investigation are in accordance with these views, superficial corneal opacity being found with equal frequency in all areas, and keratitis accompanied by dryness of the conjunctiva being recorded most often in areas where the carotene intake is low, irrespective of the incidence of cirrhosis of the liver and its effects upon vitamin A metabolism.

Tongue

The data given in Table 11 show that, in the presence of riboflavin deficiency, the changes in the tongue customarily attributed to nicotinic-acid deficiency, namely redness, oedema, hypertrophy or atrophy of the mucosa, can appear in populations having ample supplies of this nutrient, and that signs commonly considered to be the result of riboflavin deficiency, e.g. colour changes, occur more frequently in association with a low intake of nicotinic acid than when nicotinic acid is plentiful in the diet. These observations prompt the suggestion that a factor additional to lack of these two nutrients is involved in the production of such pathological conditions of the tongue.

Red, sore-looking tongues were found significantly less often in Soragbemi, where the intake of animal protein was high, than elsewhere, and signs commonly attributed to nicotinic-acid deficiency were often found where nicotinic acid is plentiful but where the intake of animal protein is very low, e.g. in Kontagora. It is suggested, therefore, that the tongue changes found in poorly nourished communities result from the lack of some factor, possibly tryptophan (Anonymous, 1950) present in animal protein, in addition to a quantitative deficiency of riboflavin and nicotinic acid, or to a disturbed relationship between protein and the vitamin B complex. Goldberger & Tanner (1921), already 30 years ago, believed that tryptophan might be the anti-pellagra vitamin.

Clinical signs presented by the Warri traders

The diets of the traders provide ample amounts of all nutrients except aneurin and riboflavin. The calculated mean daily intakes *per caput* of aneurin (0·34 mg/1000 Cal.) and riboflavin (0·46 mg/1000 Cal.) are less than those recommended by the (U.S.A.) National Research Council (1948) for moderately active men and women, weighing 139 lb. (0·61 mg riboflavin and 0·50 mg aneurin/1000 Cal.).

Keys *et al.* (1945) found that young men on diets providing 3300 Cal., 0·19 mg aneurin and 0·29 mg riboflavin/1000 Cal. did not show any physical signs of deficiency over periods up to 161 days. Yet the traders frequently suffer from nasolabial seborrhoea, abnormal vascularity of the conjunctiva and tenderness of the calves. There is good evidence for the assumption that these signs are due to a low intake of aneurin and riboflavin, and it is concluded that the amounts of these nutrients ingested by the traders are not enough to cover all the requirements of daily life and inherent individual variability.

Other signs that present themselves frequently in the traders fall into two groups: first, a sign that was recorded as often in the traders as in the peasants, namely pigmentation of the sclera; and secondly, a group of signs that, although recorded frequently in the traders, do not appear among them as often as among the peasants, namely folliculosis, atrophic skin, tropical ulcers, thickening of the conjunctiva, fissuring of the tongue, gingivitis and dental caries. It is concluded that pigmentation of the sclera is unlikely to be nutritional in origin, particularly as its incidence increases with age.

Of the other signs presented by the traders, which cannot be attributed, as a result of this investigation, to any specific nutritional deficiency, it is believed that folliculosis, atrophic skin and thickening of the conjunctiva are due to long-continued minor trauma, e.g. at pressure points for folliculosis, and bright sunlight and 'glare' for thickening of the conjunctiva. Trauma and secondary infection play a part in the aetiology of tropical ulcers, and congenital factors in the aetiology of fissuring of the tongue and their effects are difficult to separate from those of undernutrition.

It is concluded that all these signs are largely the result of long-continued minor traumata, but this does not exclude the possibility that deficiency of B-complex vitamins, and particularly of riboflavin, may exaggerate the effects of trauma.

Gingivitis was recorded frequently in all the samples of the population, but was found less often in the south than in the north. This is perhaps due to the more regular use of 'chew-sticks' by the southern natives rather than to lack of any nutrient.

Other clinical features

Evidence is not available to explain the higher incidence of rickets in the south than in the north. Dental caries was found less often in the fishermen than in the other peasant groups, and this may be the result of the large amounts of calcium in their diet.

Diffuse neuralgic pains, dyspepsia, emotional instability and easy fatigue are symptoms which cause much trouble to both medical and administrative officers. Elsom, Lewy & Heublein (1940), Williams, Mason & Smith (1939), Williams, Mason, Wilder & Smith (1940), Williams, Mason, Smith & Wilder (1942) and Keys *et al.* (1945) have all attributed these features to a low intake of aneurin. That such symptoms are more common in the southern peasants than in the northern tribes is in accord with this view.

The relationship between the mean weights of the population samples, their protein intake and the calorie value of their diets

The maintenance of body-weight in adults depends upon the balance between calorie intake and energy expenditure, but the body-weight attained by an individual is influenced by the calorie value of the diet and its protein content, provided the supply of other nutrients is adequate (Cannon, 1948; Sherman, Pearson & Bal, 1950).

Hynes, Ishaq & Verma (1946) and Verma, Dilwali & Thomson (1947) recorded the increase in weight that followed the addition of animal protein to the diets of Indian Army recruits, whose diets had been previously vegetarian. These findings have been taken to add 'one more bit of evidence for the presence of the "elusive animal protein factor"' (Anonymous, 1948; Aitken, 1949), and further evidence suggests that this factor may be identical with vitamin B_{12} (Anonymous, 1949; Wetzel, Fargo, Smith & Helikson, 1949).

The data here presented show that the greatest mean weights are attained by the populations that eat most animal protein. The Soragbemi fishermen have a mean weight of 119 lb. in spite of a calorie intake lower than that of any other group. The mean weight of the grain-eating populations (Bida, Kontagora and Zuru farmers) is only 116 lb. despite intakes of calories, total protein and vitamins of the B-complex greater than those of the fishermen. The lowest mean weight was found in the Illu farmers, whose daily consumption of protein was only 43 g. With plentiful amounts of mixed protein and calories the mean weight of the Warri traders was found to be 136 lb. These findings provide further evidence in favour of the existence of the 'animal protein factor'. It is necessary to point out, however, that the high calcium intake of the fishermen may be of importance in their development. Sherman & Pearson (1947, 1948) showed that symptoms suggestive of calcium deficiency may appear when animal protein is added to a diet, if the calcium content of the diet is kept constant.

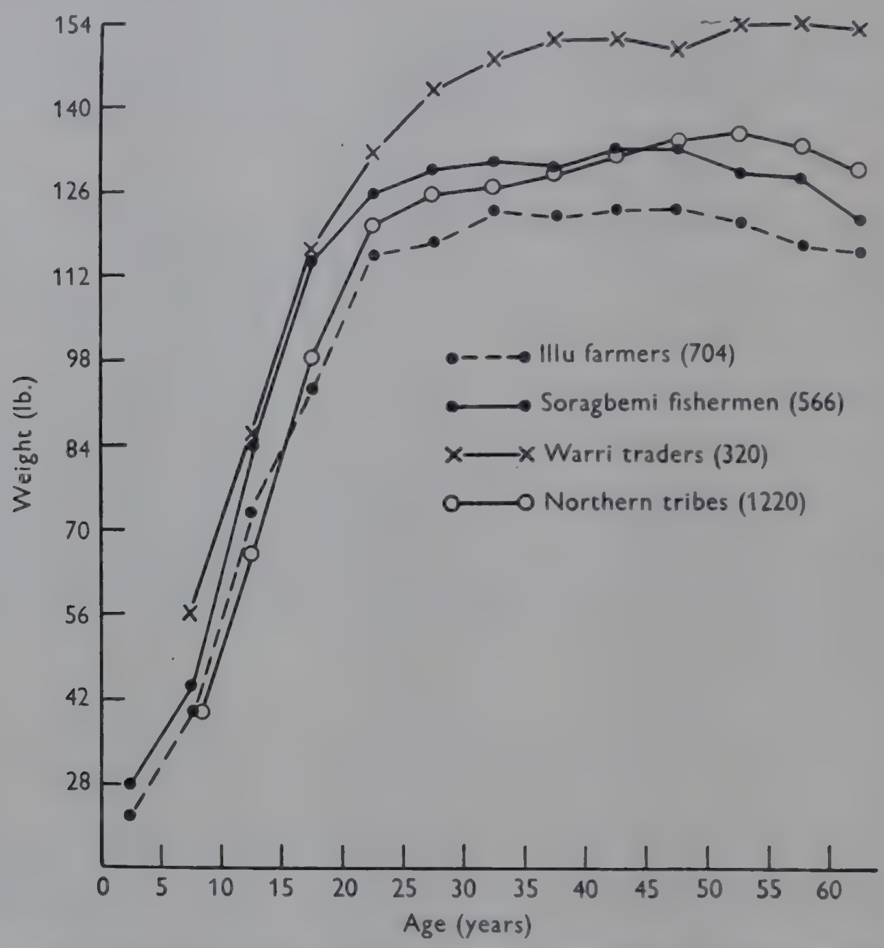

Fig. 1. Mean weights in 5-year age groups of male Nigerian peasants and traders. Figures in parentheses indicate numbers studied.

It should be noted that the weights of the northern tribes are less than those of the Soragbemi fishermen until between 40 and 50 years of age (Figs. 1 and 2), and thereafter they exceed those of the fishermen: also, the falling-off in mean weight that occurs in all samples of the population in the older age groups is more marked in the Soragbemi fishermen and Illu farmers than in the northern tribes. These weight records, considered in conjunction with calorie values of the diets, are in accordance with the views of Clements (personal communication) that 'the fall-off in body-weight in the older age groups occurs in those populations where the calorie intake is below optimum'.

The fishermen have a lower intake of B-complex vitamins than the grain-eating tribes, and yet have better general development and suffer less from cirrhosis of the liver than any other group (Table 8). Drill & Loomis (1948) showed that a high intake of animal protein can compensate to some extent for a chronic deficiency of B-complex vitamins in the prevention of functional and histological derangement of the liver.

It appears to be possible that such a compensatory mechanism is responsible, not only for the low incidence of cirrhosis among the fishermen, but also for their good general development.

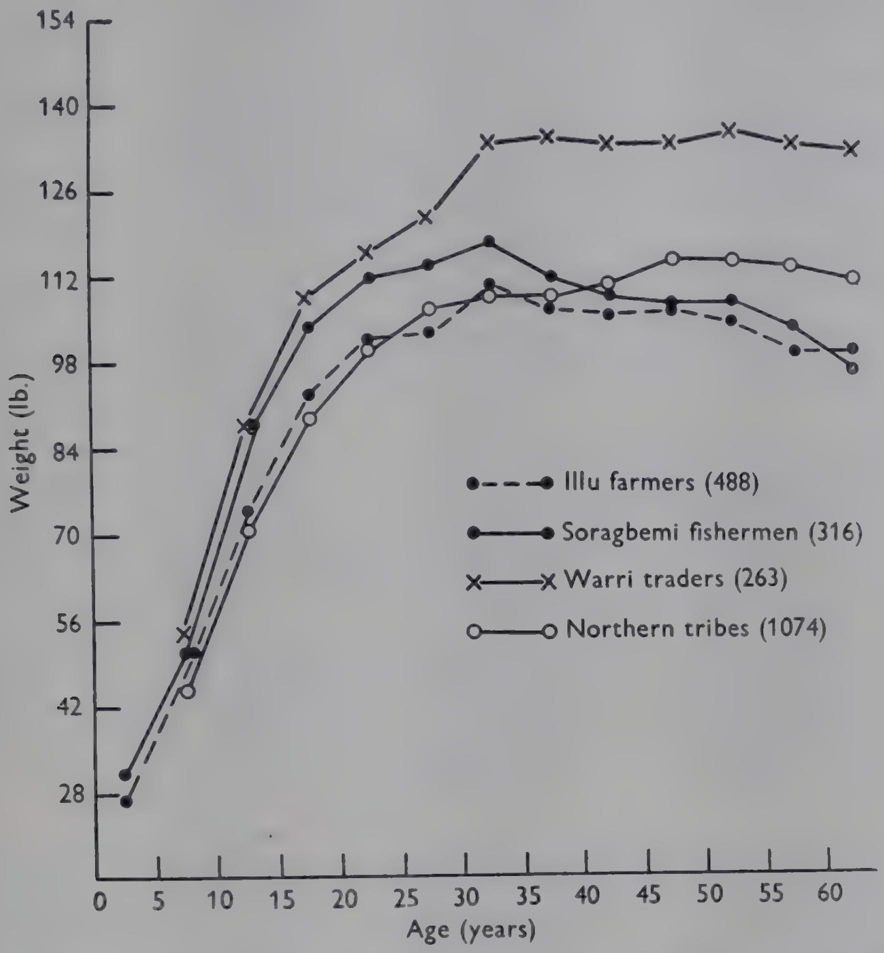

Fig. 2. Mean weights in 5-year age groups of female Nigerian peasants and traders. Figures in parentheses indicate numbers studied.

SUMMARY

1. An account is given of a nutritional survey carried out in Southern Nigeria in 1949 and 1950.

2. The methods employed to determine the nutrient intake and nutritional status of the population are described, and the findings are presented and compared with those of a previous investigation carried out in Northern Nigeria in 1947 and 1948 (Nicol, 1949).

3. The following conclusions are drawn regarding the effects of specific vitamin deficiencies:

(*a*) Vitamin A deficiency results in corneal opacity associated with dryness of the conjunctiva, staring hair and hypochromotrichia, general xerosis and follicular hyperkeratosis, 'elephant skin', 'crackled' skin and night-blindness.

(*b*) Riboflavin deficiency results in superficial corneal opacity unassociated with dryness of the conjunctiva, increased vascularity of the conjunctiva, angular stomatitis, nasolabial seborrhoea, and permanent gooseflesh.

(*c*) Aneurin deficiency leads to symptoms such as diffuse neuralgic pains, dyspepsia, emotional instability and undue proneness to fatigue and to tenderness of the calves.

(*d*) Deficiency of nicotinic acid results in 'pebbling' of the filiform papillae of the tongue.

4. Folliculosis, atrophic skin and proliferative lesions of the conjunctiva were not attributed to any specific deficiency. Long-continued minor traumata are probably the most important causes of these lesions, but deficiency of B-complex vitamins, and of riboflavin in particular, may exaggerate the effects of trauma. Pigmentation of the sclera is not the result of nutritional deficiency.

5. Evidence suggests that lack of a factor present in animal protein, possibly tryptophan, in addition to deficiencies of riboflavin and nicotinic acid, is responsible for the lesions of the tongue so commonly found among undernourished tropical communities.

6. Data are presented from which it is deduced that 1·37 mg riboflavin (0·011 mg/lb. body-weight; 0·46 mg/1000 Cal.) and 1·02 mg aneurin (0·34 mg/1000 Cal.) are not sufficient as daily allowances to cover all the requirements of daily life and inherent individual variability.

7. Consideration of the mean weights and nutrient composition of the diets of samples of the Nigerian population was taken to supply evidence in favour of the existence of a growth-promoting 'animal protein factor'.

8. Certain other clinical features are discussed.

I am grateful to the Director of Medical Services, Nigeria, for permission to publish this paper, and to Dr A. P. Meiklejohn, of the Department of Medicine and Dr R. Passmore, of the Department of Social Medicine, University of Edinburgh, for advice, criticism and help.

I am indebted to Mr D. R. Rosevear, Deputy Chief Conservator of Forests, Nigeria, for help with the botanical and zoological classifications.

Mr John Bull, of the Warri Hospital staff, rendered valuable assistance.

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The Nutrition of the Young Ayrshire Calf

7. The Biological Value of Gelatin and of Casein when given as the Sole Source of Protein

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(*Received* 27 *March* 1951)

Mature ruminants, by virtue of the extensive microbial population of their digestive tracts, are probably almost independent of any variation in amino-acid composition of their protein supply. Degradative and synthetic processes in the rumen result in mixtures of amino-acids qualitatively similar, despite marked differences in the dietary mixture given, reaching the tissues. Such independence is contingent upon a vast microbial population. It could be assumed that during the short period after birth when rumen development is negligible the young calf would require certain amino-acids in its diet. The work presented in this paper was designed to test the assumption that the young ruminant animal is as dependent on a dietary source of amino-acids as simple-stomached animals. For this purpose diets containing gelatin or casein were given to calves.

It has been known since the experiments of Edwards & Balzac (1832) that gelatin is not a sufficient source of protein, and the concept of essential amino-acids probably commenced with Escher's (1876) experiments with gelatin supplemented with tyrosine. The biological value of gelatin itself has not been determined, but a preparation of pork cracklings containing 33·5 % collagen and 25·7 % elastin gave a value with rats of 25 (Mitchell, Beadles & Kruger, 1927). Casein is a fairly adequate source of protein for the rat, having a biological value of 69 (Kon, 1928; Beadles, Quisenberry, Nakamura & Mitchell, 1933; Kik, 1938; Hughes & Hauge, 1945). The amino-acids limiting the utilization of casein have been shown to be methionine and cystine (Beadles *et al.* 1933; Kik, 1938). Table 1 summarizes the amino-acid composition of these two proteins and the percentage deficit of amino-acids calculated according to the method of Block & Mitchell (1946–7) using dried whole egg as a standard. By using dried whole egg as a standard it is not intended to imply that this protein can be used as a standard for measurement of the amino-acid requirements of the calf. The table is merely meant to indicate the large differences between the composition of gelatin and casein and a protein that has been considered quite adequate in rat nutrition.

From Table 1 it can be seen that casein is deficient in cystine and that gelatin, owing to its very high content of glycine, is an extremely unbalanced protein besides being devoid of tryptophan and deficient in tyrosine, cystine, methionine and the leucines.

Table 1. '*Essential' amino-acid composition of gelatin and casein, and the percentage deviation from the corresponding values for whole-egg proteins*

Amino-acid	Amino-acid content (%)			Percentage deviation from corresponding values for whole-egg proteins	
	Gelatin	Casein	Whole egg	Gelatin	Casein
Arginine	8·7	4·2	6·4	+ 36	−34
Histidine	0·9	3·0	2·1	− 57	+43
Lysine	5·8	7·9	7·2	− 19	+10
Tyrosine	0·7	6·9	4·5	− 84	+53
Tryptophan	0·0	1·2	1·5	−100*	−20
Phenylalanine	2·1	5·6	6·3	− 66	−11
Cystine	0·1	0·3	2·4	− 96	−87*
Methionine	0·8	3·5	4·1	− 78	−15
Cystine + methionine	0·9	3·8	6·5	− 86	−42
Threonine	2·0	4·1	4·9	− 59	−16
Leucine	3·1	9·9	9·2	− 66	+ 8
Isoleucine	1·7	6·5	8·0	− 79	−19
Valine	2·8	6·7	7·3	− 62	− 8
Glycine	24·0	2·1	—	—	—

* First limiting amino-acid.

EXPERIMENTAL

Plan of experiment

Four young Ayrshire calves were used as experimental animals. They were purchased in the market at the age of 2–4 days and were gradually accustomed to the diet used in the preliminary period. The plan of the experiment is given in Table 2.

Table 2. *Treatment of experimental animals*

Calf no.	Diet given (l./day)	Period				
		Adjustment (6 days)	Preliminary (12 days)	First experimental (12 days)	Recovery (8 days)	Second experimental (12 days)
		Diet				
16	4·8	D.S.M.	D.S.M.	Casein	D.S.M.	Gelatin
17	4·4	D.S.M.	D.S.M.	Casein	D.S.M.	Gelatin
18	4·8	D.S.M.	D.S.M.	Gelatin	D.S.M.	Casein
19	4·4	D.S.M.	D.S.M.	Gelatin	D.S.M.	Casein

D.S.M.: dried skim milk.

It will be noted that each calf received both the casein and the gelatin diet following a period during which it had been given dried skim-milk as the source of protein. The composition of the three diets is shown in Table 3.

The diets were prepared according to methods previously described (Blaxter & Wood, 1951*a*), the glyceryl monostearate and egg lecithin being used as emulsifying agents. Daily supplements of DL-α-tocopheryl acetate were given throughout. During the periods in which casein and gelatin were given, 19 ml. of a yeast extract prepared by the method of Macrae, El-Sadr & Sellers (1942) and 2 mg riboflavin were given daily. All diets were prepared at intervals of 2–3 days and stored at 5° in an immersion cooler. At low temperatures the gelatin diet was a solid jelly. It was given, as were all the diets, in a liquid form at 37°. In order to minimize difficulties in the interpretation of the results each calf was given a constant allowance of the diet throughout the experiment. Calves nos. 16 and 18 were given 4·8 l./day and calves nos. 17 and 19, 4·4 l.

Table 3. *Composition of the three diets*

Ingredient	Diet		
	D.S.M.	Gelatin	Casein
Lard (g/l.)	42·2	42·7	42·7
Glucose (g/l.)	38·1	65·0	65·0
Spray-dried skim milk (g/l.)	47·4	—	—
Casein* (g/l.)	—	—	18·9
Gelatin† (g/l.)	—	17·7	—
Arachis oil (g/l.)	3·3	3·3	3·3
Vitamin A‡ (i.u./l.)	6600	6600	6600
Vitamin D‡ (i.u./l.)	660	660	660
Yeast extract (ml./calf/day)	—	10	10
Mineral mixture no. 4§ (g/l.)	3·8	10·6	—
Mineral mixture no. 5‖ (g/l.)	—	—	10·6
DL-α-tocopheryl acetate (mg/calf/day)	50	50	50
Cholesterol (g/l.)	—	0·1	—
Lecithin (g/l.)	—	0·3	0·3
Glyceryl monostearate (g/l.)	—	0·7	0·7
Riboflavin (mg/calf/day)	—	2	2
Fat (%)	4·6	4·6	4·6
Nitrogen (%)	0·266	0·263	0·266
Cal./l. (calculated)	771	772	772
Cal. present as protein (percentage of Cal.)	11·9	11·9	11·7

D.S.M.: dried skim milk.

* The casein was commercial light white casein purchased from Messrs Prideaux Milk Foods Ltd.

† The gelatin was the highest grade commercial gelatin purchased from Messrs Richard Hodgson and Sons Ltd.; it was very light in colour and was free from elastin and heavy-metal impurities.

‡ Contained in the arachis oil.

§ Mineral mixture no. 4 was the same as that used in previous experiments (Blaxter & Wood, 1951*a*, *b*).

‖ Mineral mixture no. 5 had a lower phosphorus content to allow for the phosphorus present in the casein.

The calves were harnessed for the collection of urine and faeces, and were confined to metabolism crates as previously described (Blaxter & Wood, 1951*a*). Nitrogen metabolism was studied in each animal and included partition of the urinary nitrogen as well as determination of nitrogen balances. Respiratory-exchange determinations were made using the apparatus and technique of Blaxter & Howells (1951). Records of the health, gain in weight, pulse rate and general well-being were also kept. The analytical methods used have been described by Blaxter & Wood (1951*a*, *b*).

RESULTS

Body-weight changes

Fig. 1 shows the mean changes in body-weight on transference of the calves from the dried skim-milk diet to the casein or gelatin diet. Table 4 summarizes these data, expressed as daily gains or losses in weight, calculated by linear regression analysis.

All gains in weight of the calves when they were receiving the dried skim-milk diet or the casein diet were very low indeed. This was clearly due to the low level of protein in the diet, for, in earlier experiments (Blaxter & Wood, 1951*c*) in which a similar diet containing more dried skim-milk protein and less glucose was given in equivalent amounts, far greater gains in body-weight occurred.

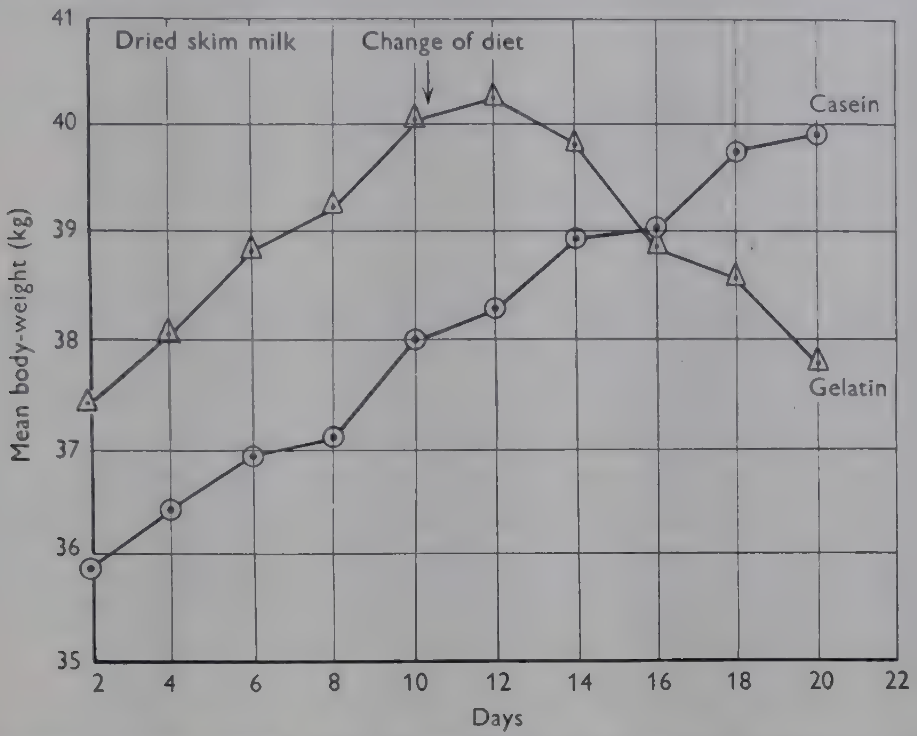

Fig. 1. The effect of diets containing dried skim-milk proteins, casein or gelatin on the mean body-weight of the calves. ⊙, calves given first the dried skim-milk diet and then the casein diet; △, calves given first the dried skim-milk diet and then the gelatin diet.

Table 4. *Mean gains or losses of the calves in body-weight in g/day*

		Period			
Calf no.	Initial weight (kg)	Preliminary (D.S.M. diet)	First experimental	Recovery (D.S.M. diet)	Second experimental
16	35·40	+248	+257 (casein)	+250	−212 (gelatin)
17	36·30	+210	+204 (casein)	+255	−239 (gelatin)
18	36·45	+377	−189 (gelatin)	+298	+200 (casein)
19	32·65	+234	−443 (gelatin)	+320	+204 (casein)

D.S.M.: dried skim milk.

Apart from the recovery period, each calf that received the higher daily allowance of milk gained more than its pair-mate, or, when the gelatin diet was given, lost less. The mean change in weight resulting from an increase in the allowance of diet from 4·4 to 4·8 l. can be evaluated from Table 5.

It will be noted from Table 5 that the animals given the casein diet gained less weight than those given the dried skim-milk diet, and that when these same animals were given the gelatin diet they could not maintain their body-weight and lost severely. Statistical analysis of their weight changes showed that the loss in weight with the gelatin diet was very highly significantly different from the gains that occurred when dried skim-milk diet or casein diet was given. The difference in gain between calves given the casein diet and those given the dried skim-milk diet was not significant.

Table 5. *Mean daily changes in weight of the calves in g/day, classified according to experimental treatment*

Level of feeding (l./day)	Protein source Dried skim milk	 Casein	 Gelatin
4·4	+222	+204	−341
4·8	+313	+278	−201
Mean	+268	+241	−271

During the recovery period the two calves that had been given the gelatin diet and had lost considerable weight gained more than those that had been given the casein diet in the preceding period. The animals previously given casein gained 252 g, those that had previously received gelatin, 307 g. The difference was significant at odds of 16 : 1, suggesting that the calf compensates for periods of malnutrition. Such a conclusion, however, may not be correct for, judging by the calves' appearance, a slight dehydration occurred during the periods when gelatin was given, and the more rapid gain probably reflects an increase in tissue hydration which took place on transference to the dried skim-milk diet.

The digestibility of the diets

The mean apparent digestibility of the dry matter, fat, and total nitrogen as well as the calculated 'apparent digestibility' coefficients of the dietary energy are given in Table 6. The standard errors of the means were derived from analysis of variance.

Table 6. *Summary of apparent digestibility coefficients of the diets with their standard errors of the mean*

Diet	Dry matter	Total fat	Total nitrogen	Calories
D.S.M.	90·7 ± 1·48	84·3 ± 2·63	79·6 ± 1·94	84·4 ± 2·56
Casein	87·5 ± 1·48	77·4 ± 2·63	77·4 ± 1·94	84·6 ± 2·56
Gelatin	85·4 ± 1·48	76·2 ± 2·63	63·5 ± 1·94	82·2 ± 2·56

D.S.M.: dried skim milk.

The digestibility of all the dietary components was low. This was probably due to the low level of protein in the diet, as is shown graphically in Fig. 2, where results for previous experiments involving dried skim-milk (Blaxter & Wood, 1951 *a–c*) are combined with those obtained in the preliminary period of the present experiment. It will be noted that a decrease in the percentage of protein derived from dried skim milk led to a marked decline in the apparent digestibility of the fat and protein of the diets. Part of this decline was possibly due to the presence of a metabolic component in the faecal excretion of both fat and nitrogen.

The apparent digestibility of both the fat and dry matter was reduced when the animals received the gelatin diet. On statistical analysis, however, the effects were only significant at a value of P between 0·1 and 0·05. The apparent digestibility of the total nitrogen of the gelatin diet was, however, significantly smaller than that of the dried skim-milk diet. The true digestibilities of the nitrogen were 90·5, 87·3 and 85·3 for the dried skim-milk, casein and gelatin diets, respectively. These results are of some interest, for it has been concluded by Morris & Wright (1935), Hutchinson & Morris (1936) and Swanson & Herman (1943) that a low apparent digestibility is inevitably associated with a high biological value. To quote Morris & Wright: 'It is not unreasonable to assume that absorption from the gut might be stimulated when inadequate rations are fed.' The true and apparent digestibilities of the dietary nitrogen in the present experiment, which refer to proteins of both high and low biological value (*vide infra*) do not confirm such a relationship. The cause of the high biological value in the experiments quoted above is in fact the low digestibility of the protein and consequent lower plane of protein nutrition rather than vice versa as the above workers have inferred.

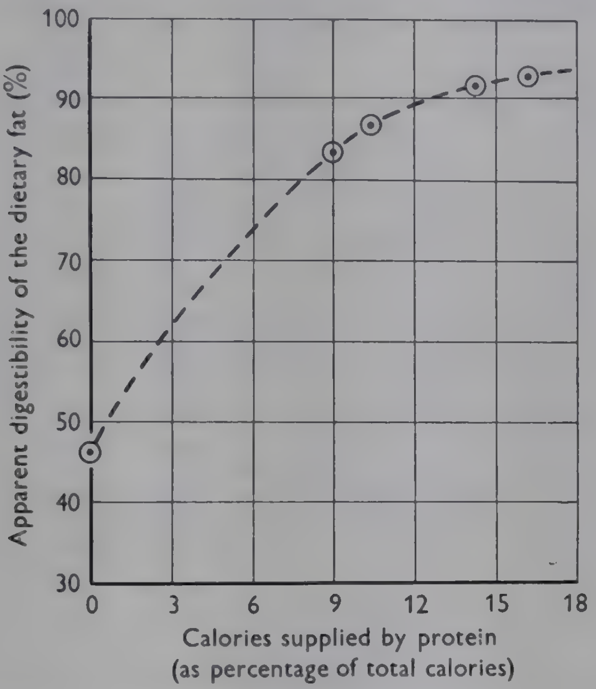

Fig. 2. The relation between the protein content of the diet and the apparent digestibility of the dietary fat.

The last column of Table 6 shows the percentage of the calorie intake that did not appear in the faeces. These values are low because the digestibility of fat was low. Nevertheless, the calculated number of calories apparently digested by any calf was higher than its basal metabolism determined at the same time (*vide infra*). In only one 2-day subperiod of the experiment was the apparent uptake of dietary energy from the gut low enough to be exceeded by the directly determined basal metabolism, and this subperiod for calf no. 19 during the period in which gelatin was given has been omitted in calculating the mean results.

Energy metabolism and pulse rate

The heat production of the calves, calculated from their respiratory exchange, was used to calculate the endogenous metabolism, using the previously determined factor of 1·90 mg endogenous nitrogen/basal Cal. (Blaxter & Wood, 1951*a*).

Table 7. *Heat production of the calves determined 15 h after feeding, calculated from respiratory-exchange determinations*

Calf no.	Diet	Heat production (Cal./24 h)	Heat production (Cal./kg/24 h)
16	D.S.M. (1)	1704	45·47
	Casein	1818	45·33
	D.S.M. (2)	1881	45·75
	Gelatin	1974	47·34
17	D.S.M. (1)	1713	44·66
	Casein	1830	44·85
	D.S.M. (2)	1431	37·69
	Gelatin	1805	43·98
18	D.S.M. (1)	1771	43·84
	Gelatin	1962	49·91
	D.S.M. (2)	1828	46·69
	Casein	2010	47·75
19	D.S.M. (1)	1590	44·54
	Gelatin	1230	39·66
	D.S.M. (2)	1706	51·08
	Casein	1680	47·67
Mean	D.S.M. (1)	—	44·63
	Casein	—	46·40
	Gelatin	—	45·22
	D.S.M. (2)	—	45·05

D.S.M.: dried skim milk. (1) preliminary period; (2) recovery period.

Table 7 summarizes the heat production of the calves.

Two low values were encountered: one for calf no. 17 in the recovery period, the other for calf no. 19 in the period in which gelatin was given. In both instances there had been a short history of anorexia or diarrhoea with a concomitant reduction in the amount of dietary energy apparently digested. In view of the instability of the calf's heat production during inanition (Blaxter & Wood, 1951*b*) such an occurrence is quite understandable. The mean heat production of the calves given 4·8 l. of the diet each day was 46·52 Cal./kg/24 h and of those given 4·4 l./day, 44·50 Cal./kg/24 h. The difference between these values was statistically significant, confirming previous findings (Blaxter & Wood, 1952) that the plane of nutrition affects the heat production of the calf when it is determined 15 h after the last feed. In this connexion it should be noted that the present mean figures for basal metabolism were greater than those observed when calves were on nitrogen-free diets and subsequently subjected to short fasts (43·1 Cal./kg/24 h (Blaxter & Wood, 1951*a*)), this again being due to the effect of the previous calorie intake.

Pulse rates were recorded throughout the experiment twice daily with an 11 h interval coinciding with feeding times. The daily records tended, however, to be variable. The mean data are shown in Table 8.

Table 8. *Mean pulse rates of the calves*

Calf no.	Level of feeding (l./day)	Pulse rate (beats/min)		
		D.S.M. diet	Gelatin diet	Casein diet
16	4·8	109·8	111·5	100·0
18	4·8	106·5	124·0	115·2
17	4·4	88·8	116·5	100·2
19	4·4	90·7	111·2	103·2
Mean	—	98·9	115·8	104·4

D.S.M.: dried skim milk.

Pulse rates were always much higher when the gelatin diet was given than when casein was the protein source, and higher on the casein than on the dried skim-milk diet. Analysis of variance of these data showed that the dietary differences were significant ($0{\cdot}01 < P < 0{\cdot}05$). It was also found that the mean pulse rates were inversely correlated with the changes of body-weight, and with the biological values of the three proteins (*vide infra*). The reasons for this relationship are dealt with later. The difference in mean pulse rate between calves given 4·8 l. and those given 4·4 l. of the diets was also statistically significant, the means being 111·1 and 101·8, respectively.

Nitrogen metabolism

The mean data relating to the nitrogen balances of each calf are presented in Table 9.

Table 9. *Mean nitrogen balances of the calves*

Calf no.	Level of feeding (l./day)	Diet	N intake (g/day)	Faecal N (g/day)	Urinary N (g/day)	N balance (g/day)
16	—	D.S.M. (1)	12·75	2·68	4·43	+5·64
	4·8	Casein	12·63	2·46	6·64	+3·53
	—	D.S.M. (2)	12·12	3·53	5·97	+2·62
	—	Gelatin	12·54	3·37	12·48	−3·30
17	—	D.S.M. (1)	11·69	2·06	4·14	+5·48
	4·4	Casein	11·66	3·17	6·12	+2·37
	—	D.S.M. (2)	11·10	4·61	4·72	+1·78
	—	Gelatin	11·13	2·79	9·27	−0·92
18	—	D.S.M. (1)	12·75	2·06	3·67	+7·01
	4·8	Gelatin	12·85	4·26	10·81	−2·22
	—	D.S.M. (2)	12·12	2·37	5·51	+4·24
	—	Casein	12·49	2·61	5·51	+4·37
19	—	D.S.M. (1)	11·69	3·12	4·51	+4·05
	4·4	Gelatin	10·98	5·89	9·46	−4·37
	—	D.S.M. (2)	11·10	4·01	4·07	+3·01
	—	Casein	11·67	2·98	4·39	+4·32
Mean, irrespective of feeding level		D.S.M. (1)	12·22	2·48	4·19	+5·55
		Casein	12·11	2·81	5·66	+3·64
		Gelatin	12·10	4·30	10·50	−2·70
Standard error of mean		—	—	±0·477	±0·449	±1·62

D.S.M.: dried skim-milk. (1) preliminary period: mean of five 2-day subperiods, except for calf no. 19 where value is the mean of four 2-day subperiods; (2) recovery period: mean of two 2-day subperiods.

Analysis of variance showed that there were no significant differences due to treatment as far as faecal nitrogen excretion was concerned, the high mean value for gelatin being largely due to one high value obtained with calf no. 19. The effect of diet on urinary nitrogen excretion was very highly significant ($P<0{\cdot}001$) as was the effect on nitrogen balance ($P<0{\cdot}01$). The effect on urinary nitrogen excretion is shown in Fig. 3. This was largely due to the very high urinary nitrogen excretion of the calves given gelatin. Nevertheless, the difference in the excretion of urinary nitrogen between the calves given casein and those given dried skim-milk was statistically significant at odds of 17 : 1.

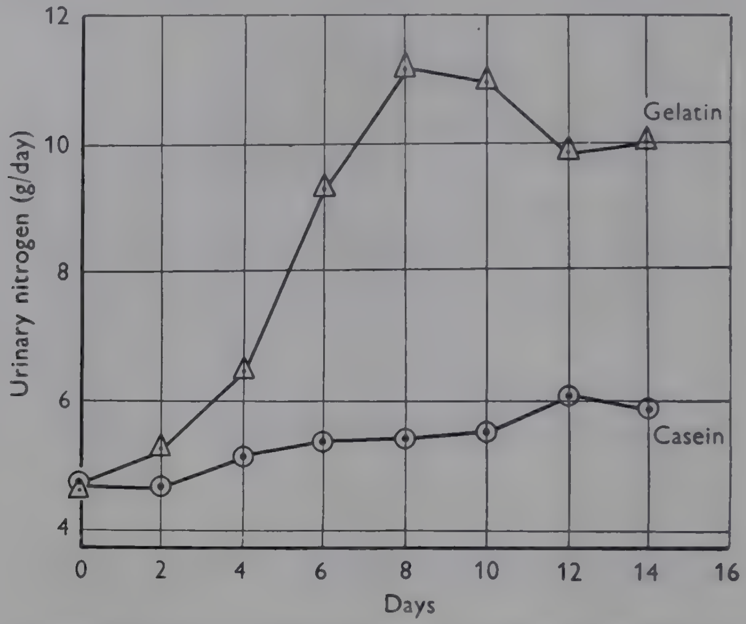

Fig. 3. The effect of transference from a diet containing dried skim-milk proteins to diets containing casein or gelatin on the mean excretion by calves of nitrogen in the urine. The excretion at 0 days represents the excretion of nitrogen when the dried skim-milk protein diet was given.

All balances were of course low, since all the diets were protein-deficient irrespective of the nutritional adequacy of the protein sources employed. It is of some interest, however, that despite this partial deficiency of protein the animals given dried skim-milk or casein gained in weight, though at a low rate. When the nitrogen balances of the calves were related to their gains in body-weight the following equation was found:

$$NB = 0{\cdot}0128\,G + 0{\cdot}898,$$

where NB is the nitrogen balance in g/day and G is the gain in weight in g/day. This regression was statistically highly significant ($P<0{\cdot}01$). The slope of the equation indicates that a gain in body-weight of 100 g corresponds to a gain of 1·28 g nitrogen. This value was much lower than the value obtained as the result of a similar analysis of results obtained with diets containing 14 % or more protein (Blaxter & Wood, 1951*c*), where the following equation applied:

$$NB = 0{\cdot}0262\,G + 0{\cdot}703.$$

In this instance a 100 g gain in body-weight corresponds to a gain of 2·62 g nitrogen.

The difference between these two estimates (2·62 ± 0·122 less 1·28 ± 0·127) is statistically highly significant, indicating that the animals given the low-protein diets in the present experiment stored considerably less nitrogen/100 g body-weight gain than those given rations less deficient in protein. A larger part of the gain in weight of the former animals must, therefore, have been body fat. This was confirmed by statistical analysis of the body-weight gains of the calves in relation to the energy content of their diets. It was found that more energy was required per 100 g gain in body-weight with the calves given low-protein diets. The regression equations were:

$$\begin{array}{ll} \text{normal diets} & G=0{\cdot}326DC-577{\cdot}5, \\ \text{low-protein diets} & G=0{\cdot}2283DC-439, \end{array}$$

where G represents the gain in body-weight in g/day and DC the total energy intake (Cal./day) less the energy excreted in the faeces (Cal./day). The regressions were both statistically highly significant ($P<0{\cdot}001$ for the equation relating to normal diets and $0{\cdot}01>P>0{\cdot}001$ for that relating to the low-protein diets). The reciprocals of these equations were calculated by transformation of the original regression equations, the regression coefficient of G being the reciprocal of the regression coefficient of DC These were:

$$\begin{array}{ll} \text{normal diets} & DC=3{\cdot}07G+1771, \\ \text{low-protein diets} & DC=4{\cdot}38G+1921. \end{array}$$

The regression constants in these two equations represent the 'digested Cal.' required per g gain in body-weight, and the intercepts of the equation represent the theoretical maintenance requirements of the calves in terms of digested Cal./day. The statistical analysis of these results is given in Table 10.

Table 10. *The statistical significance of the inverse regression functions showing that the calves given low-protein diets required more energy/100 g gain in body-weight than those given diets normal in protein*

Type of diet	No. of observations	'Digested Cal.'/g gain*	Maintenance requirement in 'digested Cal.'/day*
Normal protein	22	3·07 ± 0·131	1771 ± 97
Low protein	8	4·38 ± 0·474	1921 ± 129
Difference	—	1·31 ± 0·492	150 ± 162

* The standard errors were calculated by analysis of variance using gain in weight as the independent variable.

There was no significant difference between the two estimates of maintenance requirement. The mean estimate is in agreement with the maintenance requirement determined by respiration calorimetry (Blaxter, 1952). The difference between the two estimates of energy required per g of gain in body-weight was statistically significant. This agrees with the data presented earlier. The data do not permit the calculation of the calorific value of the retained material, since the fat, water and ash values associated with the gains are not known.

Biological value of the proteins

Biological values were calculated from the nitrogen balances and the basal energy exchanges of the calves, making the assumption (Blaxter & Wood, 1951*a*) that the endogenous metabolism of nitrogen in the calf is proportional to its basal heat loss (Table 11).

Table 11. *Calculated biological values for the calves of dietary protein*

Calf no.	Dried skim milk	Casein	Gelatin
16	89·4	72·0	19·6
17	91·6	74·4	39·7
18	97·3	80·6	29·3
19	90·7	88·1	4·4
Mean	92·3	78·8	29·5

Dried skim milk had the highest biological value, that for casein was intermediate and for gelatin the lowest. The differences between the means were statistically highly significant. The high value obtained for calf no. 19 when given casein is anomalous. It was this animal that showed the greatest loss of nitrogen when given the gelatin diet, and the subsequent high value obtained when casein was given may reflect the increased retention of nitrogen following severe depletion. Though this may be an explanation of the high value, it does not provide a reason for omitting it in calculating the mean.

It is of some interest to compare these values with those obtained for other animals. Table 12 shows such a comparison with values summarized by Block & Mitchell (1946–7).

Table 12. *Biological value of proteins for man*, rat* and calf*

	Value for			Relative values (D.S.M. = 100)		
Protein	Calf	Rat	Man	Calf	Rat	Man
Dried milk	92	84	74	100	100	100
Casein	79	69	58	85	82	78
Gelatin	29	25	—	32	30	—

D.S.M.: dried skim milk.
* From Block & Mitchell (1946–7).

The values obtained with calves were all higher than those obtained with rats, which were in turn higher than those obtained with men. As shown in the second part of Table 12, the relative values obtained when dried skim milk was taken as equal to 100, were, however, very similar, irrespective of species. It appears, therefore, that the nutritive values of proteins as diverse in amino-acid content as gelatin and casein are relatively the same in the calf as in simple-stomached animals.

The reason why the biological values determined with calves were higher than those determined with other species was probably that the level of protein in their diets led to a greater degree of protein deficiency than occurred in rat or man. The results do not imply a species difference in the nutritive value of the three proteins used, but rather they indicate that the experiments with rats, calves and men are not necessarily comparable with regard to the degree of protein undernutrition employed.

The distribution of urinary nitrogenous metabolites

The mean results obtained from analysis of the urine for nitrogenous metabolites are given in Table 13. These means refer to the last 6 days of each period except for calf no. 19 when given the gelatin diet and calf no. 18 when given the casein diet. A single sample of urine in these periods had to be rejected and the mean daily excretion was measured over 4 days. Albumin nitrogen, creatinine nitrogen, allantoin nitrogen and the total purine nitrogen were unaffected by the dietary source of protein. There was a small decline in the excretion of purine nitrogen as uric acid accompanied by an increase in excretion as purine bases. These changes, however, involved only small quantities of nitrogen and were not sufficiently large to result in any marked change in the total excretion of purine. The major part of the increase in urinary nitrogen excretion was in the urea, ammonia and amino-nitrogen fractions. These three fractions alone accounted for nearly 90 % of the increased nitrogen excretion by the calves when given the gelatin diet. Creatine nitrogen also increased when either the gelatin or the casein diet was given.

Table 13. *Summary of mean daily excretion by the calves of urinary metabolites during the last 6 days of each period*†

(in mg nitrogen/day)

Nitrogenous metabolite	Diet			Standard error of difference between means
	D.S.M.	Casein	Gelatin	
Urea and ammonia	2010·8	2997·8	6326·8	±378***
Albumin	128·8	143·5	149·9	±33·4 N.S.
Amino-N	268·9	282·0	711·5	±82·7***
Creatinine	407·8	378·4	384·0	±16·5 N.S.
Creatine	296·0	330·9	499·3	±54·7*
Purine base	93·6	146·0	252·1	±27·0*
Uric acid	69·9	70·4	50·0	± 4·2*
Allantoin	599·1	518·7	530·6	±12·5 N.S.

D.S.M.: dried skim milk.
† Last 4 days for calf no. 19 on gelatin diet and calf no. 18 on casein diet.
* Significant at $P=0{\cdot}05$.
*** Significant at $P=0{\cdot}001$.
N.S.: Not significant.

These results are quite compatible with the contention that there was an increase in the deamination of dietary amino-acids when the calves were given either gelatin or casein as a sole source of protein. That this increase was due to failure of the protein to supply essential amino-acids and not due to a shortage of dietary energy is shown, first, by the fall in uric-acid excretion, since an increase occurs in energy starvation (Blaxter & Wood, 1951*b*) and, secondly, by the fact that with the exceptions previously mentioned, the basal metabolism of the calves was not lower than in the preliminary period when dried skim milk was given. A fall in the basal metabolism of the calf would have occurred had energy starvation ensued (Blaxter & Wood, 1951*c*). The constancy of creatinine elimination and of purine elimination despite such large changes in nitrogen metabolism, suggests that the endogenous metabolism of Folin (1905) does represent a reliable base-line for use in such studies.

General observations

All calves tended to become a little weak, as judged by a diminished playfulness in their metabolism cages and their general lethargy. Hair loss was considerable throughout, increasing in severity when the casein diet was given. As previously mentioned, mild alimentary disturbances were invariably present. When the calves were given the gelatin diet, they refused some of it after 8–10 days, and they tended to become dehydrated. On these occasions more water was added to their feed and in only one instance (calf no. 19) was it necessary to resort to the injection of a normal salt solution to restore tissue hydration. When the gelatin diet was given, pigmentation of the tongue developed in this calf, and the whole oral cavity became a dark magenta colour. Nicotinic-acid deficiency was suspected in this tryptophan-deficient calf, but no additional nicotinic acid was given in view of the metabolic relationships between tryptophan and nicotinic acid. There was no response to additional riboflavin (2 mg/day) and the condition slowly improved as the experimental period progressed. A similar condition, though not so severe, was noted in calf no. 16 when transferred to the gelatin diet. The exclusion of nicotinic acid from diets containing large quantities of casein does not produce any abnormalities in young calves (Johnson, Wiese, Mitchell & Nevens, 1947).

At slaughter nothing abnormal was noted in the carcasses of the calves. Serums were collected at this time and the serum proteins determined by sodium-sulphate fractionation. A non-protein fraction and three protein fractions were obtained using three concentrations of Na_2SO_4 (0, 159, 192 and 380 g/l.). These fractions differ from those proposed by Howe (1921), and were used as a result of fractionation studies on cattle serums by the method of Majoor (1946, 1947). Table 14 summarizes the results.

Table 14. *Concentration of serum-protein fractions of the calves at slaughter*

(g/100 ml.)

Na_2SO_4 concentration (g/l.)	Fraction of serum protein	Calf no.					Normal calf given adequate colostrum
		16	17	18	19	Mean	
0–159	‘γ globulin’	1·84	1·09	1·15	1·05	1·26	2·14
159–270	‘α+β globulin’	1·84	1·81	1·65	1·59	1·72	1·85
Above 270	Albumin	1·97	2·29	1·67	1·94	1·97	2·64
	Total	5·67	5·19	4·57	4·62	4·95	6·61

Results obtained with a normal calf aged 6 days given cow's whole milk are included for comparison. The serum-protein concentration was low in all the experimental calves, the reduction being in both the globulin and the albumin fraction. In adult cattle the total serum-protein concentration is approximately 7–8 g/100 ml., this higher figure being accounted for mainly by a greater concentration of globulins. The low globulin content of the blood of the present calves may be partly accounted for by lack of proteins transmitted from the dam in the first few hours of life, but the low albumin content is clearly related to the inadequate level of protein nutrition.

DISCUSSION

Since the young calf can store body nitrogen and grow when given a protein known to be of fair nutritive value for the rat and yet will lose body nitrogen and weight when given an inadequate protein, it obviously is dependent on its diet for at least a part of its amino-acid supply. That it may be as dependent as the rat or man is demonstrated by the close similarity between the biological values obtained for the three different species for a good protein (dried skim milk), a fair protein (casein) and a very poor protein (gelatin). These results, to our knowledge, are the first demonstration that farm ruminants require dietary sources of amino-acids for metabolic functions involving nitrogen metabolism. The results apply, however, only to the calf reared under conditions in which development of an adult rumen function is unlikely to have occurred, and thus, considered together with the fact that the adult ruminant does not react in a similar manner, provide additional confirmation of Zuntz's (1891) hypothesis of rumen function.

This finding has a number of implications; it raises the question of the protein values of the calf meals and gruels used for calf rearing; it implies that the utilization and requirement of the young calf for a number of other dietary essentials may differ markedly from the utilization and requirement of the adult (cf. Wiese, Johnson, Mitchell & Nevens, 1947, Johnson *et al.* 1947), and it implies that during the early part of the calf's life, when rumen function has commenced but has not reached its maximal synthetic capacity, the calf may still be liable to dietary shortages of amino-acids.

The fact that losses in weight occurred when gelatin was given suggests that tryptophan—the first limiting amino-acid of gelatin—is required for maintenance of nitrogen equilibrium and body-weight. Where such a loss in weight occurs on a diet sufficient in energy it is obvious that the metabolism of the animal must increase. The higher pulse rates found when gelatin was given suggest that an increase in heat production did occur. That this was due to the increase in the heat increment of feeding and not to an increase in basal metabolism is implied by the fact that the basal metabolic rate was not significantly greater when the calves were given gelatin. The conclusion that the heat increment of feeding is greater in the animal given a diet deficient in essential amino-acids is in agreement with the general conclusions of the other workers, as recently reviewed by Kleiber (1945–6).

SUMMARY

1. Experiments have been made in which four calves were given dried skim-milk proteins, casein or gelatin as the sole source of protein. These diets were adequate in other respects but contained only 11·9 % of their total energy as protein and were thus deficient in protein.

2. The mean daily changes in body-weight of the calves were +268, +241 and −271 g for the dried skim-milk, casein and gelatin diets respectively.

3. There were no significant differences between the diets in respect of the apparent digestibility of dry matter, fat, nitrogen or calories, but the digestibility of the dietary fat was related to the protein content of the diet.

4. Basal heat production determined after 15 h fasting was not affected by the dietary differences. Pulse rates were 98·9, 104·4 and 115·8 when protein was supplied by dried skim milk, casein and gelatin, respectively. These differences were statistically significant.

5. Urinary nitrogen excretion rose when the calves were transferred from the dried skim-milk diet to the casein diet and was greatly increased when gelatin was given. The mean daily nitrogen balances were: dried skim milk +5·55, casein +3·64 and gelatin −2·70 g. The differences between these balances were statistically significant.

6. It was shown that the storage of nitrogen/g gain of body-weight was much lower in calves given protein-deficient diets than in those given sufficient protein. The protein-deficient animals required more energy/g gain in body-weight. It was therefore concluded that their gains of body-weight contained less protein and more fat than those of calves given more normal diets.

7. The biological values of the three proteins were 92·3 for dried skim milk, 78·8 for casein and 29·5 for gelatin. These values were higher than those found with rats, probably as a result of a relatively lower level of protein nutrition with the calves. When the biological values were related to dried skim milk taken as 100, there were no differences between the biological values obtained on casein and on gelatin as between calf, rat and man.

8. There was no significant change in the excretion of creatinine nitrogen, purine nitrogen or protein nitrogen. There was, however, a small change in the distribution of the urinary purine. Excretion of urea, ammonia, amino-nitrogen and creatine, however, increased when casein or gelatin rather than dried skim milk was given.

9. The results of the experiment are briefly discussed in relation to the hypothesis that the microflora of the digestive tract plays an important part in the protein metabolism of ruminants. It is concluded that, unlike the adult ruminant, the young calf is as dependent on its diet for a source of essential amino-acids as man, dog, or rat.

Our thanks are due to Mr D. Paterson for his care of the experimental animals, and to Miss C. Samson and Miss G. Breckenridge for their technical assistance.

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Observations on the Vitamin Metabolism of the Common Fowl

1. The Partition of Free and Combined Riboflavin in the Tissues of the Laying Hen

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(*Received* 9 *April* 1951)

A considerable amount of work has been carried out on the estimation of the minimal requirements for riboflavin by laying hens, both for egg production and for hatchability, but comparatively little is known of the part of riboflavin in egg production.

Common & Bolton (1946) and Common, Rutledge & Bolton (1947) showed that the riboflavin content of the blood serum of immature pullets receiving injections of oestrogens increased to between ten and twenty times the pre-treatment value. In the latter paper they demonstrated that differences of the same order existed between serums of normal laying and non-laying hens.

Hertz, Dhyse & Tullner (1949) confirmed for plasma the increase in riboflavin following oestrogen treatment, and showed that this increase was greater when the diet contained adequate riboflavin than when a diet poor in riboflavin was given.

It has also been shown that the increase in serum riboflavin evoked by oestrogen treatment consists entirely of an increase in the free vitamin, the two naturally occurring combined forms, flavin-mononucleotide (FMN) and flavin-adenine-dinucleotide (FAD) being unaffected (Bolton, 1950). Examination of the blood serums of laying hens and of the albumen and yolk of their eggs showed that almost all the riboflavin in the blood serum was in the free form and that 85 % of the riboflavin in the egg was also present as the free vitamin in both albumen and yolk (Bolton, 1951).

It therefore became of interest to ascertain the partition of riboflavin in the oviduct, since this organ forms the link between the blood-stream and the egg. This paper sets out the results obtained from an investigation of the partition in two parts of the oviduct, the magnum, where the yolk of the egg receives its layers of albumen, and the shell gland. Three other tissues were also examined: liver, kidney and heart. Liver and kidney are known to be rich in the vitamin, and from previous work (Bolton, 1947) the concentration in all three is known to reflect the level of intake by young chicks. A similar observation has been made for the livers of immature pullets (Hertz *et al.* 1949). The yolk of the egg is formed in the ovary and takes several days to grow to its full size; this part of the egg has therefore been disregarded in the present study.

EXPERIMENTAL

Birds. Eleven Brown Leghorn hens bred at this Centre were used. The birds were housed separately in wire cages and were offered 90 g daily of an all-mash diet. It was suspected that the differences to be measured might be relatively small. A diet low in riboflavin was therefore used to render the differences more easily detectable. The all-mash diet was made up as follows, in parts by weight: maize meal 37, Sussex-ground oats 38, wheat offal 10, fish meal 4, decorticated extracted groundnut meal 6, cod-liver oil 1, ground limestone 3·5 and salt 0·5. To each 100 lb. of mash 20 g manganese sulphate were added. Water was supplied *ad lib.*, but no grit was given. The birds consumed all but a few g of the mash each day; when offered 100 g/day they did not consume any more, and the mash allowance may therefore be taken as *ad lib.* feeding. Each meal was assayed separately for riboflavin by the method of Kodicek & Wang (1949). The riboflavin content of the final diet as computed from these assays was 1·3 μg/g food; this concentration was found by Norris, Wilgus, Ringrose, Heiman & Heuser (1936) to represent the minimal requirement for egg production.

Methods. The course of depletion was followed by determining the riboflavin content of the albumen and yolk of the eggs laid. These contents decreased in the course of a month from 90 and 80 to 55 and 40 μg riboflavin per egg, respectively, but did not fall below these last two values, thereby indicating that depletion was complete.

All hens received the low-riboflavin diet for at least a month. Each hen was weighed, killed and then bled to remove as much blood from the tissues as possible. The stage in the ovulatory cycle was assessed as accurately as possible by visual examination during dissection, and the five tissues were removed, weighed and stored in the refrigerator overnight to become thoroughly cold.

The partition of riboflavin into the free vitamin, FMN and FAD was carried out by a macromodification of the method of Bessey, Lowry & Love (1949), since the microfluorimeter necessary for the original method was not available. A portion of 5 g of tissue was taken for the initial maceration and all analyses were completed within 48 h of the death of the bird.

RESULTS

Table 1 contains the body-weight and lengths of magnum and shell gland for each hen. Table 2 gives the mean content of the three riboflavin fractions of the five tissues examined in each hen in terms of μg total content and of concentration/g tissue.

DISCUSSION

Liver, kidney and heart. All the hens had received the low-riboflavin diet for at least a month, and depletion of the bird, as indicated by the content of riboflavin in the egg, was as complete as the diet permitted, yet riboflavin was present as the free vitamin in all the tissues. This suggests that a metabolic demand for the vitamin in the unphosphorylated form exists in the body, a view substantiated by the similar results of Bessey *et al.* (1949) on the tissues from rats given a low-riboflavin diet.

Table 1. *Body-weight and lengths of magnum and shell gland for each hen*

Hen no.	Body-weight (g)	Length of Magnum (mm)	Length of Shell gland (mm)	Stage of ovulatory cycle
L. 3113	1617	306	38	Nearing ovulation
L. 3103	1530	306	46	Nearing ovulation
L. 3004	2040	299	51	Nearing ovulation
L. 3012	1628	241	63	Egg just into shell gland
L. 3101	1324	299	53	Egg just into shell gland
L. 3001	1777	264	51	Egg just into shell gland
L. 3035	1512	234	48	Fully formed egg in shell gland
L. 3096	1578	216	84	Fully formed egg in shell gland
L. 3021	1855	267	59	Ovum atretic
L. 3109	1585	259	61	Ovum just into magnum
L. 3094	1480	221	38	Going out of lay

Table 2. *Mean total riboflavin contents of the tissues of the hens*

Tissue	Free riboflavin* (μg)	Free riboflavin* (μg/g)	Flavin-mononucleotide* (μg)	Flavin-mononucleotide* (μg/g)	Flavin-adenine-dinucleotide* (μg)	Flavin-adenine-dinucleotide* (μg/g)	Total* (μg)	Total* (μg/g)
Liver	39 ± 5	1·2 ± 0·2	45 ± 6	1·3 ± 0·2	581 ± 36	17·2 ± 1·0	665 ± 41	19·7 ± 1·0
Kidney	9 ± 2	0·9 ± 0·2	20 ± 4	1·6 ± 0·3	200 ± 17	17·0 ± 1·1	229 ± 19	19·5 ± 1·1
Heart	3 ± 1	0·6 ± 0·1	6 ± 1	1·2 ± 0·2	56 ± 7	10·2 ± 0·9	65 ± 8	12·0 ± 1·0
Magnum	43 ± 9	2·0 ± 0·3	28 ± 5	1·4 ± 0·2	69 ± 7	3·4 ± 0·2	140 ± 6	7·0 ± 0·5
Shell gland	6 ± 1	0·39 ± 0·07	6 ± 1	0·37 ± 0·04	39 ± 1	2·6 ± 0·2	49 ± 6	3·3 ± 0·2

* Value with its standard error.

The amounts of free riboflavin present in the kidney and heart are so small that they can contribute little to the demand for the free vitamin as the albumen is laid down. On the other hand, the liver contains substantial stores of free riboflavin and cannot be so disregarded. When the results are expressed on a percentage basis, it is seen that the partitions of the riboflavin into free vitamin, FMN and FAD are almost identical in each of the three tissues. Moreover, when the contents are considered according to the stage in the ovulatory cycle, a relationship is not observed, although the number of results is insufficient to rule out the liver on this count alone.

However, when all the information is taken together, it suggests that, though the riboflavin contents of liver, kidney and heart reflect the level of intake and to that extent may serve as an ultimate reserve for egg production, these tissues are not involved in the day-to-day metabolism of riboflavin in egg formation.

Magnum and shell gland. In the process of egg formation, the ovum spends about 3 h in the magnum and 20 h in the shell gland. The albumen is secreted during the passage of the ovum through the magnum, and Conrad & Scott (1942) have shown that albumen accumulates in this tissue during the intervals between the passage of eggs.

It is therefore of interest to find that the magnum contains the largest amount of free riboflavin, whether this is expressed as μg/g tissue or as total amount in the tissue.

It has been mentioned above that the albumen of each egg from the depleted hens contained 55 μg riboflavin. Of this about 44 μg were present as the free vitamin. The results have therefore been examined to see whether, in fact, the amounts found in the magnum alone were sufficient to permit this secretion of 44 μg. The results are set out in Table 3; those for hen no. L. 3021 are given along with those for the two birds that had fully formed eggs in the shell glands, since she had laid within a few hours of her death.

Table 3. *Free riboflavin in the magnum of the laying hen at different stages of the ovulatory cycle*

Condition of hen	Free riboflavin (μg)
Nearing ovulation (hens nos. L. 3113, L. 3103 and L. 3004)	84
Egg just into shell gland (hens nos. L. 3012, L. 3101 and L. 3001)	41
Egg in shell gland, fully formed (hens nos. L. 3021, L. 3035 and L. 3096)	25

The difference in content of free riboflavin between those birds about to ovulate and those with an egg just passed into the shell gland amounts to 43 μg, which corresponds to the amount of free riboflavin in the egg.

The percentage composition of the shell gland suggests that this tissue may also be involved in riboflavin secretion, but the total amount present is so small that it would play a minor part compared with the magnum. An examination of the results failed to reveal any differences related to the stage in the ovulatory cycle, and it is concluded that this part of the oviduct plays a negligible, if any, part in the secretion of riboflavin into the egg.

Though the total number of birds examined was not enough securely to establish the premise, the results obtained suggest that the magnum is the only investigated tissue involved in the day-to-day mechanism affecting the amount of riboflavin in the egg. Immediately before ovulation the content of free riboflavin is high; after the egg has passed through the organ the content is reduced by an amount equal to the content of free vitamin in the egg. It is therefore suggested that the riboflavin in the albumen is secreted at the same time as the albumen layer itself and probably passes to the egg with the albumen.

SUMMARY

1. The partition of riboflavin into free vitamin, flavin-mononucleotide and flavin-adenine-dinucleotide in the liver, kidney, heart, magnum and shell gland of laying hens has been determined.

2. In liver, kidney and heart, about 85 % of the vitamin is present as the dinucleotide, with slightly more mononucleotide than free riboflavin accounting for the rest.

3. In the magnum there is 50 % more free riboflavin than mononucleotide and three times as much dinucleotide as mononucleotide.

4. The shell gland has three-quarters of its riboflavin present as dinucleotide, the rest being equally divided between the free form and the mononucleotide.

5. It is suggested that the magnum is the tissue involved in the day-to-day mechanism affecting the amount of riboflavin in the egg.

The author thanks Dr A. W. Greenwood, the Director of this Centre, for his advice and criticism, and Miss D. R. Mitchell for assistance both in the care of the birds and in the analyses.

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The Use of Chicks for the Biological Assay of Members of the Vitamin B Complex

2. Tests on Natural Materials and Comparison with Microbiological and Other Assays

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(*Received* 21 *June* 1951)

In a previous paper Coates, Kon & Shepheard (1950) suggested methods for the biological assay of nicotinic acid, riboflavin, pyridoxin, pantothenic acid and folic acid with chicks. The accuracy of the methods was determined for preparations of the pure vitamins. The present paper reports their application to assaying the same vitamins in various natural materials, and the results are compared with those found by microbiological or other means.

EXPERIMENTAL

Substances tested

While the technique of the chick assays for the B-vitamins was still in the experimental stage, the materials chosen for study were those, such as milk and yeast products, already well studied by other methods. However, the chick tests were primarily designed for comparison with results obtained by microbiological techniques, which, mainly because of their speed and simplicity of operation, are increasingly being used for routine determinations of the vitamins. Since they are applied in particular at this laboratory to experiments on the synthesis of vitamins by the micro-organisms of the alimentary tract, it was thought advisable to examine certain gut-content materials by the chick method.

The following twelve substances were tested for one or more of the vitamins listed; the first three were issued by the Vitamin B Subcommittee of the Accessory Food Factors Committee (Medical Research Council) for tests which were part of a co-operative research on methods organized by that Subcommittee:

(1) Flour of 85 % extraction, prepared under the supervision of the staff of the Research Association of British Flour Millers, through the courtesy of Dr T. Moran, Director.

(2) Dehydrated minced beef obtained through the co-operation of Dr F. Kidd, F.R.S., then Superintendent of the Low Temperature Research Station, Cambridge.

(3) Dried food yeast; a roller-dried preparation of *Torula utilis* supplied by Dr A. C. Thaysen from the Chemical Research Laboratories, Teddington.

(4) Dried brewer's yeast.

(5) A yeast extract.

(6) Dried beer solids.

(7) Dried skim milk; a commercial sample of good quality.

(8) Sow's milk; a pooled, freeze-dried sample of milk from several sows, prepared as described by Davis, MacVicar, Ross, Whitehair, Heidebrecht, Braude, Coates, Henry, Kon, Thompson & Wilby (1950).

(9) Dried spinach-beet; a mixture of spinach-beet leaves freshly picked, freed from stalk, immediately dried in a current of warm air at about 45° and milled before use.

(10) Dried rat faeces, collected from normal stock-colony rats and dried at about 70°, then milled finely.

(11) Dried whole rumen contents, kindly supplied by our colleague Dr J. W. G. Porter, collected from a fistulated cow over several weeks, spread on stainless steel trays, dried in a current of warm air at about 45°, then milled and the whole sample remixed mechanically before use.

(12) Fish solubles, kindly supplied by Dr J. A. Lovern of the Torry Research Station, Aberdeen; a concentrate of a watery extract obtained in the course of manufacture of herring oil by the 'cook-and-press' method.

Standards

For the work with chicks commercially pure samples of the vitamins were used throughout, since the quantities involved were large. For microbiological tests the Medical Research Council Provisional Standards were used when available; synthetic pteroylglutamic acid (Lederle Laboratories Inc.) was used as folic-acid standard in both methods. The two sets of standards were compared microbiologically and corrections were made when necessary, the results being expressed in terms of microbiological standards.

Chick assays

The procedure followed was essentially as described previously (Coates, Kon & Shepheard, 1950). The inclusion of the test materials in the diet presented difficulties. Most of the materials were bulky and of low potency, so that it was sometimes necessary to replace as much as 20 % of the diet by the test sample to get a measurable response. The basic components of the diet were then adjusted to preserve the nutritive value of the basal diet as far as possible unchanged. Where the sample, such as dried faeces or flour, consisted mainly of fibrous or carbohydrate material, it was added at the expense of the dextrin. If it contained an appreciable amount of protein, as in dried milk, some of the casein was omitted from the basal diet. Certain materials for which a chick assay was required were of too low potency to give a measurable response even when forming 20 % of the diet. Such samples were fortified before testing by the addition of a known amount of the vitamin to be assayed. The limits of error of such an assay were inevitably large, and it could hence be used only for detecting marked divergences from the results of the corresponding microbiological test. This technique was used in the assay of riboflavin in flour and of riboflavin, pantothenic acid and folic acid in rumen contents.

In early assays the diet was given in powdery form, but it was given granulated as soon as the advantages of that method of feeding were realized (Coates, Kon & Shepheard, 1950). The amount of water used in granulation was varied to suit the material tested, but fairly satisfactory granules were obtained in all instances.

Whenever possible four doses of the test material were assayed against four doses of standard, with about fifteen birds in a group, although occasionally the amount of test material available was insufficient for so many. Frequently results for one or more of the groups could not be included because their response was in the maximum or minimum range or because in the groups with low doses many birds had died before the end of the test.

Microbiological assays

Technique

Assay methods. The methods used were according to published procedures or modifications of them described below.

Recovery tests. In a number of instances, shown in the tables of results, extracts were prepared, from samples of the material studied, with and without addition of a known amount of the vitamin under test. From the measured differences in the potencies of the two extracts the percentage recovery of the added vitamin was calculated.

Internal and external standards. The potency of each test extract was compared with that of a dilute solution of the relevant vitamin (external standard). Also in a number of instances, before carrying out the final dilution step in the preparation of an extract, two equal portions were taken and to one of them was added a known amount of the vitamin. Both portions were then equally diluted with water to provide the solutions for assay. A comparison of the potencies of these two solutions made it possible to calculate the potency of the extract in terms of an internal standard. In some assays the solutions containing internal standard were tested at fewer dose levels than those of the external standard, and results with such internal standards may hence have been less precise than those with external standards, but in all the examples reported there was broad agreement between results obtained by the two different methods.

Vitamins unstable to light. Assays for riboflavin, vitamin B_6 and folic acid were carried out by red light or in a darkened room.

Incubation. For most of the assays carried out with *Lactobacillus casei*, *Lb. arabinosus*, *Streptococcus faecalis* and *Leuconostoc mesenteroides*, the baskets of tubes, covered with towels, were incubated in an enclosed water-bath fitted with a pump to circulate the water continuously during the period of incubation. In some of the earlier assays an air incubator was used. The usual period of incubation was 60–72 h, whether responses were to be measured turbidimetrically or by titration.

General

The design of the microbiological assays was in general such that no straightforward statistical assessment of the errors of the results was possible. In some instances results were obtained by reference to both internal and external standards or by the application of a variety of extraction procedures, and for certain assays of riboflavin and folic acid by the use of two different assay organisms. In some assays we have been unable to apply a simple linear transformation to the standard-response curve. All available results are therefore presented, supported in many instances by recovery values or statements of the extent of agreement between values obtained by reference to internal with those by external standards. The problem of statistical interpretation of the results has been studied by Mr E. C. Fieller of the Mathematics Division of the National Physical Laboratory, Teddington, and his findings will be published separately. In general, there was reasonable agreement between the results quoted here, which were obtained by the usual process of 'reading-off' and those obtained by Mr Fieller's more detailed calculations. The limits of error for his results proved in most cases to be within 90–110 % of either calculated figure.

Riboflavin

Substances tested. Dried skim milk, 85 % extraction flour, dried sow's milk and dried rumen contents were assayed.

Methods of assay. The method of the Analytical Methods Committee (1946), with the medium designated (Y), was applied to dried skim milk. Five strains of *Lb. casei* were used; three were obtained by propagation from isolated colonies of *Lb. casei* A.T.C.C. 7469; the other two, L.G.N. and Y.T.C. 1, had growth characteristics different from one another and from the other three cultures.

The remaining substances were assayed with *Lb. casei* A.T.C.C. 7469 by the method of Roberts & Snell (1946) and also with *Leuc. mesenteroides* A.T.C.C. 10100 by the method of Kornberg, Langdon & Cheldelin (1948), modified by supplementing each 100 ml. of the double-strength basal medium with the following: DL-tryptophan, 20 mg; adenine, guanine, uracil and xanthine, 2 mg each; nicotinic acid, pyridoxin hydrochloride, calcium DL-pantothenate and aneurin hydrochloride, 20 μg each; *p*-aminobenzoic acid, 100 μg; biotin, 0·1 μg; folic acid, 0·4 μg. Use of the supplement slightly increased the sensitivity of the assay and resulted in a more nearly linear standard-response curve. The effect of omitting individual constituents of the supplement was not investigated.

Treatment of samples. All the samples were prepared for assay by extraction with 0·1 N-hydrochloric acid, as described by the Analytical Methods Committee (1946). From the 85 % extraction flour, extracts were prepared also by digesting the sample for 4 h with takadiastase (Parke, Davis & Co.) at pH 4·6 and the acid-hydrolysis procedure referred to above. For dried sow's milk comparisons were made between extracts treated and not treated with ether and between results obtained by direct addition of aqueous dilutions of the dried sow's milk to the assay tubes and those obtained from extracts prepared as described above.

Nicotinic acid

Substances tested. Dried rat faeces, dried brewer's yeast, yeast extract, dried beer solids and dried rumen contents were tested.

Method of assay. Lb. arabinosus 17-5 was used. For the first four substances the method was that described by the Analytical Methods Committee (1946), except that the medium was supplemented with asparagine and glutamic acid, 40 mg of each per ml. double-strength basal medium; the addition increased the responses. For the remaining substances the medium of Roberts & Snell (1946) was used, modified by omission of nicotinic acid and inclusion of riboflavin.

Vitamin B_6

Substances tested. Dried food yeast, dried beef, 85 % extraction flour and dried rumen contents were tested.

Method of assay. Saccharomyces carlsbergensis 4228 was used as described by Atkin, Schultz, Williams & Frey (1943), with the following modifications. The medium was supplemented with nicotinic acid and ammonium phosphate, as recommended by Hopkins & Pennington (1947); the final liquid content of assay tubes was 5 ml.; the tubes were autoclaved at 10 lb. pressure for 10 min, cooled in ice before inoculation and brought to 30° in a water-bath before transfer to a shaker in an air incubator.

Treatment of substances tested. Extracts were as a rule prepared by autoclaving the substances for 5 h at 20 lb. pressure with 0·055 N-sulphuric acid, as recommended by Rabinowitz & Snell (1947). For the 85 % extraction flour, 0·44 N-acid was used for the extraction, as recommended by Atkin *et al.* (1943).

Folic acid

Substances tested. Dried food yeast, dried spinach-beet leaves and dried rumen contents were tested.

Method of assay. The assays were usually made with *Lb. casei* A.T.C.C. 7469 in the medium of Roberts & Snell (1946), modified by omission of folic acid and inclusion of riboflavin. In some instances extracts were assayed also with *Strep. faecalis* Rogers in the medium of Teply & Elvehjem (1945).

In a recent publication, Koft, Sevag & Steers (1950) remark on the difficulty they encountered in preserving folic-acid solutions. We have used a stock solution with 1000 μg folic acid (Lederle Laboratories Inc.)/ml. in 25 % ethyl alcohol containing 1 % sodium bicarbonate. The stock solutions were prepared at intervals of several months and with each assay two standard solutions were included, one being diluted from the most recent stock solution and the other from an older one. In tests with *Lb. casei* or *Strep. faecalis*, we have obtained no evidence of loss of folic acid from the stock solutions after storage for 3 months at 2° or after incubation for 10 days at 30°. A stock solution containing 200 μg folic acid/ml. in M/15 phosphate buffer at pH 7·3 was unchanged by incubation for 10 days at 30° in the presence of 1 % of the preservative of Hutner & Bjerknes (1948); in the absence of preservative there was bacterial growth and a 40 % loss of folic-acid activity.

Treatment of substances tested. The routine method adopted was that of Sreenivasan, Harper & Elvehjem (1949), and involved digestion of the substance with an aqueous extract of pig kidney at pH 4·6 and then digestion with an extract of chick pancreas at pH 7·0. We departed from the published method by not autolysing our extract of chick pancreas and by carrying out the digestion of rumen contents and dried spinach-beet leaves under the preservative of Hutner & Bjerknes (1948) instead of under toluene.

Pantothenic acid

Substances tested. Dried food yeast, condensed fish solubles, skim milk powder and dried rumen contents were tested.

Method of assay. Yeast, fish solubles and dried rumen contents were assayed with *Lb. arabinosus* 17-5 in the medium of Roberts & Snell (1946), modified by the omission of pantothenic acid and inclusion of riboflavin. For the assay of skim milk powder the medium of Barton-Wright (1946) was used.

Treatment of substances tested. Extracts of yeast, fish solubles and dried rumen contents were prepared by the method of Nielands & Strong (1948), which was published after the tests had been made on skim milk powder. The milk was extracted by the variety of procedures set out in Table 1.

RESULTS

Chick tests

The results are set out in Table 2.

Microbiological tests

A summary of the mean results obtained is set out in Table 2. More detailed results are presented under the appropriate vitamin headings. The result given in Table 2 was generally the mean of all results available, but in some instances certain results were omitted, because they were not considered sufficiently precise. In all tables the results from which the means quoted in Table 2 were derived are indicated.

Riboflavin. For skim milk powder the results obtained with the five cultures of *Lb. casei* by external and internal standards were similar to one another. They are given in Table 3. For the other samples assayed (dried sow's milk, dried rumen contents and 85 % extraction flour) the results are given in Table 4.

Table 1. *Pantothenic-acid content of four natural materials, assayed microbiologically*

Substance tested	Method of extraction		Pantothenic-acid* content (μg/g)	Recovery (%)
Dried food yeast	Digestion at pH 8 with extracts of calf intestine and of chick liver		160	106
			156	73
			166	117
			148	85
		Mean	158	95
Fish solubles	As above		51	—
			79	116
			57	—
			60	—
			44	—
			47	—
		Mean	56	—
Dried rumen contents	As above		14	—
Skim milk powder	Dissolved in water		28	—
	As above, then autoclaved at 15 lb. for 15 min		30	—
			27	—
	Dissolved in water and made to pH 4·6. Heated to 100° and filtered. Digested with mylase P (British Drug Houses, Ltd.) (after Ives & Strong, 1946)		23	100
		Mean	27†	—

* Expressed as calcium pantothenate.

† Reference to internal standards gave comparable results, with mean value 24 μg/g.

Nicotinic acid. Results for the five substances assayed are set out in Table 5.

Vitamin B_6. Results for the four substances assayed are set out in Table 6.

Folic acid. Results for the three substances assayed are set out in Tables 7 and 8.

Pantothenic acid. Results for the four substances assayed are set out in Table 1.

Rat tests

In several instances results are available for rat tests carried out on some of the substances examined in the present study. The tests were done for a different purpose by Dr K. M. Henry, but have so far not been published. They are set out in Table 9 and quoted in Table 2.

Physical and chemical tests

Results are also available for physical and chemical tests done in this laboratory on certain substances. They are quoted in Table 2.

Table 2. *Content of certain B vitamins in natural materials determined with chicks, microbiologically, by physical or chemical methods and by rat assay*

Vitamin	Substance assayed	Content from				
		Chick assay		Micro-biological assay. Value (μg/g)	Other available sources	
		Value (μg/g)	True fiducial limits at $P=0·95$ (μg/g)		Method	Value (μg/g)
Riboflavin	Dried skim milk 1	19·5	18·1–21·1	18·1	Fluorimetric*	18·3
	Dried skim milk 2	21·0	19·0–23·5	Not tested	Fluorimetric*	20·1
	Dried sow's milk	7·0	5·8–8·6	6·3	Fluorimetric*	6·4
					Rat assay†	8·2
	Dried rumen contents	15·6	8·8–24·8	11·1	Fluorimetric‡	11·0
	Flour, 85 % extraction	0·50	0·16–0·83	0·7	Fluorimetric‡	0·63
Nicotinic acid	Dried rat faeces	243	155–373	191	Chemical§	181
	Dried brewer's yeast	687	470–1068	700	—	—
	Yeast extract	429	269–760	916	Chemical§	871
	Dried beer solids 1	54	27–94	163	Chemical§	160
	Dried beer solids 2	52	35–71	148	—	—
	Dried rumen contents	62	47–81	57	—	—
Vitamin B_6 (expressed as pyridoxin hydrochloride)	Dried food yeast	38	33–46	30	Chemical§	33
					Rat assay†	39‖
	Dried beef	3·3	1·7–5·1	2·9	Chemical¶	3·4
					Rat assay†	3·6‖
	Flour, 85 % extraction	2·1	1·6–2·6	1·4	Chemical¶	4·0
					Rat assay†	2·5‖
	Dried rumen contents	3·7	2·6–4·9	5·1	—	—
Folic acid	Dried food yeast	19·6	16·3–23·8	7·2	—	—
	Dried spinach-beet leaves	10·5	7·3–14·8	15·9	—	—
	Dried rumen contents	0·74	0·39–1·14	1·2	—	—
Pantothenic acid (expressed as calcium pantothenate)	Dried food yeast	150	132–172	158	—	—
	Condensed fish solubles	67	55–84	56	—	—
	Skim milk powder	41	28–55	27	—	—
	Dried rumen contents	17·6	7·9–28·4	14	—	—

* See Davis *et al.* (1950).

† See Table 9.

‡ Unpublished method of S. Y. Thompson.

§ Method of James, Norris & Wokes (1947).

‖ Combined result of two assays.

¶ Method of Brown, Bina & Thomas (1945).

Table 3. *Riboflavin content of skim milk powder, assayed microbiologically*

Designation of *Lb. casei* culture	Riboflavin content by External standard (µg/g)	Riboflavin content by Internal standard (µg/g)
A.T.C.C. 7469 isolate 1	18·8	18·0
A.T.C.C. 7469 isolate 2	18·5	19·0
A.T.C.C. 7469 isolate 3	19·8	17·4
L.G.N.	16·7	16·2
Y.T.C. 1	17·7	18·8
Mean	18·1	

Table 4. *Riboflavin content of dried sow's milk, dried rumen contents and 85 % extraction flour, assayed microbiologically*

Substance tested	Method of preparing extract	Test organism*	Riboflavin content (µg/g)	Recovery (%)
Dried sow's milk	Dissolved in water	Lb. c.	6·3	86
		L. mes.	6·8	96
	As Analytical Methods Committee (1946)	Lb. c.	7·0	125
		L. mes.	5·4	84
	As Analytical Methods Committee (1946), but extracts treated with ether	Lb. c.	7·5	106
		L. mes.	4·8	96
		Mean	6·3	99
Dried rumen contents	As Analytical Methods Committee (1946)	Lb. c.	12·3	74
		Lb. c.	13·5	—
		L. mes.	8·7	75
		L. mes.	9·7	—
		Mean	11·1	—
Flour, 85 % extraction	As Analytical Methods Committee (1946), but extracts treated with ether	Lb. c.	0·67	100
		Lb. c.	0·72	—
	Digested with takadiastase, then as Analytical Methods Committee (1946)	Lb. c.	0·67	—
		Lb. c.	0·72	—
		L. mes.	0·68	—
		L. mes.	0·70	—
		Mean	0·69	—

* Lb. c.: *Lb. casei* A.T.C.C. 7469; L. mes.: *Leuc. mesenteroides* A.T.C.C. 10100.

Table 5. *Nicotinic-acid content of five natural materials, assayed microbiologically*

Substance tested	Nicotinic-acid content by External standard (µg/g)	Nicotinic-acid content by Internal standard (µg/g)		Recovery (%)
Dried rat faeces	191	163*		—
Dried brewer's yeast	700	671*		—
Yeast extract	916	768*		—
Dried beer solids 1	163	—		—
Dried beer solids 2	145	158	Mean 148	90
	149	138		87
Dried rumen contents	56	—	Mean 57	80
	57	—		80

* These results are less precise than those obtained by reference to external standards (see p. 77) and the latter are therefore quoted in Table 2.

Table 6. *Vitamin B_6 content of four natural materials, assayed microbiologically**

Substance tested		Vitamin B_6 content by External standard (μg/g)	Internal standard (μg/g)		Recovery (%)
Dried food yeast		30	—		—
		30	—		95
	Mean	30	—		—
Dried beef		2·9	—		—
		2·8	—		102
	Mean	2·9	—		—
Flour, 85 % extraction		1·2†	—		—
		1·5	—		91
	Mean	1·4	—		—
Dried rumen contents		5·2	—	Mean 5·1	142
		5·3	—		112
		4·8	4·4		104
		5·2	5·6		94
	Mean				113

* Each substance was assayed twice, with an interval of 2 years' storage at −20° between assays.
† Extracted with 0·055 N-sulphuric acid.

Table 7. *Folic-acid content of dried food yeast, assayed microbiologically after different extraction procedures*

Method of extraction	Folic-acid content by *Lb. casei* (μg/g)	*Strep. faecalis* (μg/g)	Recovery (%)
Digestion with pig-kidney extract followed by dilution with water and filtration (Bird, Bressler, Brown, Campbell & Emmett, 1945)	3·7	3·0	—
	3·3	3·0	—
	3·3	—	—
Digestion with pig-kidney extract, filtration omitted	4·3	—	—
	4·5	—	—
Digestion with chick-pancreas extract, pH 5	6·3	5·8	—
	6·8	—	—
As above, but with larger amounts of chick-pancreas extract	6·7	—	86
Digestion with chick-pancreas extract, pH 8	6·0	—	97
Digestion with pig-kidney extract, pH 4·6, then with chick-pancreas extract, pH 7 (Sreenivasan *et al.* 1949)	6·9	—	86
	7·5	—	80

Each result is for a separately weighed portion of yeast. The highest results, 6·9 and 7·5 (mean 7·2), obtained after digestion with pig-kidney and chick-pancreas extracts, were those taken for comparison with the results of the chick assay in Table 2.

Table 8. *Folic-acid content of dried spinach-beet leaves and dried rumen contents, assayed microbiologically*

Substance tested	Method of extraction	Test organism	Folic-acid content (μg/g)	Recovery (%)
Dried spinach-beet leaves	Digestion with pig-kidney extract, pH 4·6, then with chick-pancreas preparation, pH 7·0	*Lb. casei*	16·9	128
			16·5	65
		Strep. faecalis	15·6	86
			14·6	77
		Mean	15·9	89
Dried rumen contents	As above	*Lb. casei*	1·1	—
			1·2	75
		Mean	1·2	

Table 9. *Content of riboflavin and vitamin B_6 in natural materials determined by tests with rats*

itamin	Method	Substance assayed	No. of groups: Standard	No. of groups: Test	No. of animals/group	Value (μg/g)	True fiducial limits at P=0·95 (μg/g)
oflavin	Henry, Houston & Kon (1940)	Dried sow's milk	3	2	10	8·2	7·3–9·3
amin B_6	Copping (1943)	Dried food yeast	3	2	4♂	41	30–61
			3	2	4♀	38	29–52
		Dried beef	3	2	4♂	3·3	2·1–4·4
			3	2	4♀	3·9	2·7–5·1
		Flour, 85 % extraction	3	3	4♂	2·0	1·3–2·5
			2	2	4♀	3·0	2·3–4·3

DISCUSSION

The biological assay by the chick method of members of the vitamin B complex in natural materials proved possible, although, as might be expected, the error was somewhat large, especially for samples of low potency. As the test sample was incorporated directly into the chicks' diet without any preliminary extraction or other preparatory treatment, the results are a measure of the physiological availability of the vitamins rather than their total amount.

For the microbiological tests the reproducibility by any one set technique was generally good, and the inherent error was hence comparatively small. However, a microbiological test, though reproducible, may fail to measure the activity of a vitamin as established by direct feeding test with higher animals, not only because it assays the activity for a different organism but also because the test is generally applied not to the substance itself but to an extract prepared from it. In none of the microbiological tests reported here was there any marked discrepancy between results obtained with external and with internal standards, indicating that the vitamins had the same growth-promoting potency in presence or absence of the other substances present in test extracts. Agreement between results with both types of standard therefore provides some evidence for the validity of the results. Recovery tests also, where applied, generally showed little or no loss and indicated that the procedures used were capable of estimating the different vitamins, at any rate in so far as they were available in the test extracts to the appropriate micro-organisms.

Broadly speaking, there was in our experience good agreement between the microbiological and chick tests, although there were some marked discrepancies, of which three outstanding examples were the determinations of nicotinic acid in dried beer solids and yeast extract and of folic acid in dried yeast.

The results obtained by chick assay for nicotinic acid in beer solids was on two separate occasions only about one-third of that found microbiologically and by chemical test. For yeast extract, the chick value was less than half that found by these two methods. Several explanations may be suggested to account for the differences. First, samples were prepared for microbiological and chemical test by autoclaving with N-hydrochloric acid, and this rather drastic treatment may have released some of the vitamin from a form not readily available to animals. Secondly, compounds may have been present that were as active for bacteria as nicotinic acid and were chemically measured as such, but useless to the chick, though this possibility is rather remote, not only because of the good agreement between microbiological tests on beer solids with internal and external standards (Table 5) but also because of the good agreement between the chemical measurement and microbiological assay. The third possibility is that some inhibitor or antivitamin may have been present in the beer solids and yeast extract and have interfered with proper utilization of the nicotinic acid by the chicks.

Whatever the reason for the discrepancy, it is clear that very much less nicotinic acid was biologically available to the chicks than was indicated by the microbiological and chemical techniques.

The interpretation of these comparisons is in any event complicated by the biological interrelationship of nicotinic acid and tryptophan. It is known that chicks can utilize tryptophan in place of nicotinic acid (Krehl, Sarma, Teply & Elvehjem, 1946; Rosen, Huff & Perlzweig, 1946; Briggs, Groschke & Lillie, 1946); in our experience with the diet used in the present studies 40 mg of additional tryptophan gave a growth response equivalent to that usually produced by about 2 mg nicotinic acid.

When chicks were used to assay dried yeast for folic acid the result obtained was several times higher than any found microbiologically. It is possible that the enzyme treatment in vitro, used before the microbiological test, was less effective in the breakdown of conjugated forms of folic acid than was the complicated system of enzymes in the digestive tract of the chick. Possibly also there may exist in yeast precursors or metabolites of the pteroylglutamates of greater activity to chicks than to the micro-organisms used. It is unlikely that the high result found by the chick method was due to a lack in the basal diet of some nutrient present in yeast, for satisfactory agreement between the two techniques was obtained in the assays of pyridoxin, pantothenic acid and nicotinic acid in yeast.

Though other workers (Bird *et al.* 1945; Burkholder, McVeigh & Wilson, 1945) obtained good agreement between microbiological and chick tests for folic acid on yeast, Tables 2 and 7 show that we failed to do so, whether we applied the enzyme treatments used by these authors separately or together.

Discrepancies such as those just described amply justify caution in interpretation of microbiological results and emphasize that the reactions of micro-organisms to any nutrient as present in natural materials are not necessarily those of higher animals. A microbiological assay may, however, have considerable advantages over a non-biological technique, as is shown by the measurement of riboflavin in sow's milk (see Davis *et al.* 1950). Good agreement was reached between the microbiological and chick techniques, whereas a chemical method well-tried for cow's milk gave much lower results; further investigation revealed the presence in the sow's milk of riboflavin in a form not detectable by the chemical procedure used.

Apart from these examples of marked discrepancy between microbiological and chick tests, there were several smaller differences that may have been due to imperfections in technique. Thus, the agreement between the tests for pantothenic acid by the two techniques studied was reasonably good for yeast, fish solubles and rumen contents, but for milk the chick test gave a higher value. Pantothenic acid is similar to folic acid in often occurring naturally in combined forms, from which it must be liberated by enzyme action before it becomes available to the micro-organisms used by us. It is possible that milk supplied the chicks with an essential factor absent from the basal diet or that the enzyme treatment before microbiological assay may have been less efficient than that of the chick's digestive system.

The microbiological assay of vitamin B_6 was done with *Saccharomyces carlsbergensis*, reported by Snell & Rannefeld (1945) and Miller & Baumann (1945) to respond about equally to the three forms of the vitamin, pyridoxal, pyridoxamine and pyridoxin. According to Luckey, Briggs, Elvehjem & Hart (1945) the activity for chicks of pyridoxal or pyridoxamine is less than that of pyridoxin. Hence if these compounds when present in natural materials behave in the same way as in the free form, one might expect the chick tests to yield on the whole lower results than microbiological tests. This was so in our experience with rumen contents, but not with yeast, meat and flour. The chick findings agreed, however, very well with those we obtained on rats and, except for flour, with admittedly rather unreliable chemical measurements. It is possible that the extraction before the microbiological test was not complete or that the basal diet for chicks and for rats was lacking in some factor other than vitamin B_6. Of the known factors, vitamin B_{12} was not added, but examination of the chicks used in this laboratory has shown that they carry reserves of vitamin B_{12} (Coates, Harrison & Kon, 1950). Evidence is accumulating (Stokstad, Jukes, Pierce, Page & Franklin, 1949) that the so-called 'animal protein factor' may well be of a complex nature. Whether the supply of 'animal protein factor' to the chicks used in these assays is optimum, and how best it may be supplemented if it is not, can be decided only when more is known of its identity and distribution.

Until the importance of the various factors just quoted is thoroughly investigated, they may give rise to error in any biological assay carried out by these methods. However, if the possibilities of error are borne in mind when interpreting results, assays by the chick method can contribute reliable information about the biological availability of vitamins of the B complex and can also serve as checks on the applicability of microbiological and chemical methods to estimating the requirements of higher animals.

SUMMARY

1. The methods of assaying vitamins of the B_2 complex with chicks, described by Coates, Kon & Shepheard (1950) for pure substances, have been applied to twelve natural materials, and the results have been compared with those of microbiological tests and of some chemical and rat assays as well.

2. Dried milks, rumen contents, faeces, yeast products, flour, meat, fish solubles and spinach-beet leaves were assayed for one or more of the following vitamins: riboflavin, nicotinic acid, vitamin B_6, folic acid and pantothenic acid.

3. On the whole the agreement was satisfactory, the notable exceptions being nicotinic acid in yeast extract and dried beer solids, for which the chick assay gave much lower results, and folic acid in yeast, where the reverse was true.

4. The tests show that the microbiological methods used can be applied to a wide range of natural materials. They also emphasize the need for a biological check before microbiological results are interpreted in terms of animal requirements.

We are greatly indebted to our colleagues, Dr S. Y. Thompson and Dr J. W. G. Porter, for the physical and chemical tests quoted in this paper, and we should especially like to thank Dr K. M. Henry for permission to quote the results of biological tests with rats.

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The Importance to Sheep of Frequent Feeding

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(*Received* 4 *July* 1951)

It has long been the practice of good stockmen to feed their animals 'a little and often'. This paper describes how this belief has been tested experimentally.

EXPERIMENTAL

Twenty-two Cheviot ewe hoggs were paired and divided into two equal groups which were comparable in age, body-weight and previous history. The sheep were penned singly, and to each was fed a daily ration consisting of 1 lb. chopped hay mixed with $1\frac{1}{2}$ lb. of the following concentrate mixture: yellow maize meal 57, crushed oats 15, wheat bran 14, linseed-cake meal 7, and white fish meal 7%.

In addition to these rations each animal was offered an *ad lib.* supply of water and had access at all times to both plain salt and mineral licks. This quantity of food was maintained throughout the 18 weeks of the experiment as it was considered that to increase it with increasing body-weight would serve only to complicate the experiment.

During the first part of the experiment, which lasted for a period of 9 weeks, group A was fed eight times daily at approximately hourly intervals between the hours of 9.30 a.m. and 5.15 p.m. On each occasion one-eighth of the total ration was given, i.e. 3 oz. concentrates and 2 oz. chopped hay. Group B received its daily ration as one large feed at 9.15 a.m. For the second 9-week period the treatments were reversed, group B being given the frequent treatment and group A fed once daily. The animals were weighed twice weekly throughout the experiment. Food residues were weighed, no account being taken of their composition, and the total quantity of food eaten daily was recorded. Animals on a single feed rarely left a food residue, but, on the other hand, those fed frequently commonly did this as is shown in Table 2.

RESULTS

The criteria used in the interpretation of the results are those of body-weight gains and efficiencies of food utilization. The latter refer to net efficiencies and are calculated by dividing the starch equivalent (lb.) consumed over and above maintenance requirements during each 9-week period by the body-weight gain (lb.) during that time. The results of the experiment are shown in terms of body-weight gains in

Table 1, and the body-weight changes for both groups are expressed graphically in Fig. 1.

Table 1. *Frequent versus single feeding of sheep. Body-weight gains of all animals throughout the experiment*

Group A				Group B			
	Body-weight gain (lb.)				Body-weight gain (lb.)		
Sheep no.	Period 1 (frequent feeding)	Period 2 (single feeding)	Difference due to frequent feeding	Sheep no.	Period 1 (single feeding)	Period 2 (frequent feeding)	Difference due to frequent feeding
1	7	4	3	16	1	13	12
2	9	5	4	21	2	15	13
3	9	−1	10	22	−2	14	16
4	15	5	10	18	2	16	14
5	7	3	4	15	−1	9	10
6	17	4	13	14	7	12	5
7	5	−1	6	20	6	17	11
8	10	5	5	13	1	12	11
9	4	6	−2	17	3	14	11
10	8	2	6	23	1	16	15
11	5	1	4	24	−2	13	15
Total	96	33	63		18	151	133

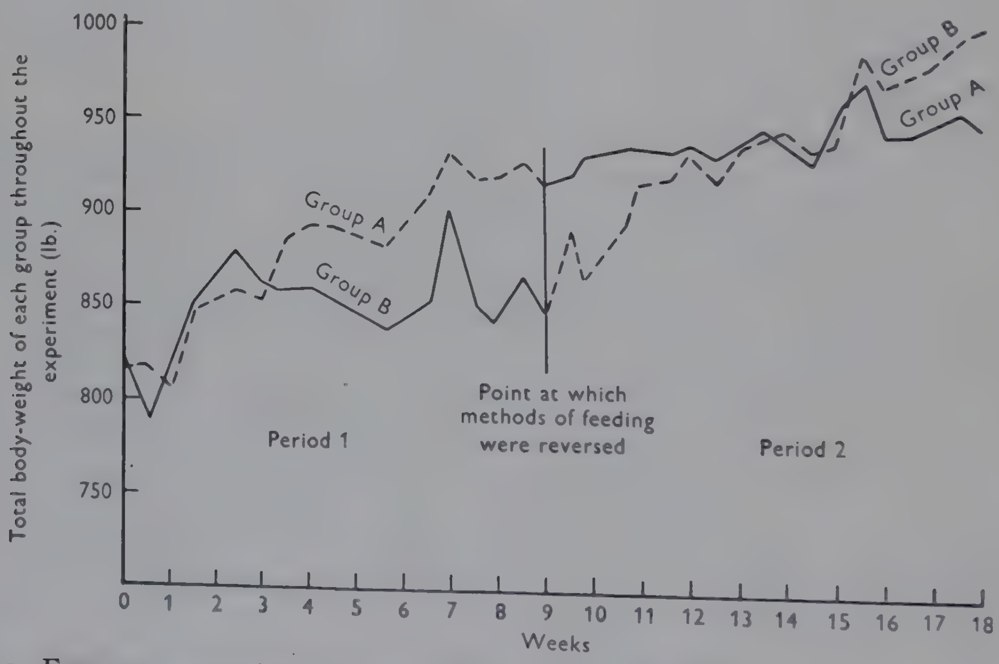

Fig. 1. Frequent versus single feeding of sheep. Total body-weight of each group during both experimental periods. – – –, frequent feeds; ——, single daily feeds.

During period 1, group A, on frequent feeding, gave a total live-weight increase of 96 lb., whereas group B, on one feed daily, increased by only 18 lb. During period 2, group A, now fed only once each day, gave a total live-weight increase of 33 lb., whereas group B, now fed frequently, gained 151 lb.

By subtracting the increase on single feeding from that on frequent feeding for each group the total increase due to frequent feeding is obtained. For group A this is

63 lb. and for group B 133 lb., a total of 196 lb. extra live-weight gain. This same total can also be arrived at by adding together the differences between the groups during the same periods. Statistical analysis has shown that these differences are highly significant ($P < 0{\cdot}001$).

This experiment suggests that not only does frequent feeding increase the rate of live-weight increase but it also causes a depression of appetite. Tables 1 and 2 show that in group A over the period of frequent feeding an average of 8·6 lb. of food was refused by each sheep, an average gain in body-weight of 9 lb. was made, and the average efficiency of utilization was 5·9. On single daily feeds in period 2 an average of only 1·4 lb. of food was rejected and a gain of only 3 lb. was made. The efficiency of utilization was much inferior, namely 15·0. In group B these figures are even more decisive. While frequently fed these animals gave an average efficiency quotient of 3·3, but when fed only once each day this figure rose to 27·5. While on frequent feeding all the animals involved consumed on an average 13·5 lb. of food for every lb. of live-weight increase. On single feeding this was increased to the very high figure of 67·5 lb. of food per lb. of increase.

Table 2. *Frequent versus single feeding of sheep. Food residues and net efficiencies of food utilization for each animal throughout the experiment*

(The net efficiencies are calculated by dividing the starch equivalent (lb.) consumed over and above maintenance requirements during each 9-week period by the body-weight gain (lb.) during that time)

Group	Sheep no.	Food residue (lb.)	Efficiency of food utilization	Food residue (lb.)	Efficiency of food utilization
		Period 1 (frequent feeding)		Period 2 (single feeding)	
A	1	0	6·7	1·3	11·0
	2	1·8	5·2	2·9	9·0
	3	5·25	4·7	0	—
	4	10·5	3·2	4·5	9·5
	5	6·75	6·4	0	14·9
	6	0	2·7	0	10·5
	7	0	8·4	1·8	—
	8	4·25	4·4	1·2	8·5
	9	59·7	9·1	3·5	7·4
	10	6·0	5·6	0	21·8
	11	0	8·6	0	42·1
	Mean	8·57	5·9	1·4	15·0
		Period 1 (single feeding)		Period 2 (frequent feeding)	
B	16	0	48·0	3·9	3·4
	21	0	23·7	9·8	2·7
	22	0	—	2·6	3·1
	18	0	25·8	7·8	2·8
	15	0	—	2·0	4·9
	14	0·7	6·8	3·8	3·6
	20	8·15	7·5	1·1	2·5
	13	0	46·2	3·8	3·6
	17	0	16·0	1·1	3·8
	23	0	46·2	2·2	2·7
	24	0	—	3·1	3·1
	Mean	0·84	27·5	3·7	3·3

It is of interest to note a further comparison. The total body-weight of all the sheep involved at the start of the experiment was 1642 lb.; at the end they totalled 1940 lb. Thus a total gain of 298 lb. was made, of which 196 lb., or 65·8 %, were due to frequent feeding, an effect equivalent to the expected live weights of about two and a half sheep or the carcass weights of four or five sheep at the end of the same feeding period if frequent feeding had not been employed.

DISCUSSION

To what may these differences be attributed? Unfortunately it is only possible, at the moment, to offer a few suggestions with little or no experimental backing. Four possibilities will be discussed.

(1) It may be that when a ration is divided into several small feeds spread over a long period of time the total surface area open to attack by micro-organisms in the rumen might be larger and/or available for a longer time per unit of microbial activity than when the same quantity and quality of food is given as one meal. In this way a more efficient coefficient of digestibility might be obtained.

(2) The work of Schalk & Amadon (1928) and Balch (1950) suggests that since food material passes into the omasum from the reticulum at a constant and not too rapid rate throughout the day, and since during a meal the heavier and more finely divided portion of the ration rapidly reaches the reticulum, then under a system of small feeds this portion will pass on and into the abomasum in a short period of time. Therefore the loss of energy due to fermentation processes will be minimal. However, if a large amount of food is ingested at one time it probably remains for longer in the rumino-reticular cavity, and this disproportionately greater delay will result in large energy losses.

(3) A completely different aspect is suggested by the third possibility. The even supply of substrate and the supposedly even production of metabolites that may occur under the frequent feed system may (*a*) give rise to an alimentary environment better suited to the growth and metabolism of micro-organisms, and (*b*) it may enable the animal to utilize the metabolites more efficiently than under the single feed system where there may be a more rapid flooding of the tissues than is compatible with an optimal rate of utilization.

(4) It is possible that the length of time spent ruminating by the animal under the two systems of management may be different and might, by the consequent differences in mechanical action upon the food, result in differences in digestibility. Continuous observations were made on the ruminating behaviour of four sheep, two from each group, over a 72 h period. The total time spent ruminating by the two frequently fed sheep was 2534 min, whereas the two singly fed sheep spent 3483 min ruminating. Further work is continuing along these lines.

It will be noticed that these four possibilities assume that frequent feeding is only effective in giving increased body-weights in ruminants. It would be of interest to know whether such is the case with single-stomached animals.

In a pilot digestibility-trial, in which it was unfortunately only possible to use two sheep, one on each treatment, results of a 10-day experimental period, following a 9-week preliminary period, gave differences in the digestibility of nitrogen of the order of 10 %, and a nitrogen balance showed a retention of 52 g nitrogen in favour of the frequently fed animal.

It was considered that these results are sufficiently suggestive to warrant an expansion of this study. At the moment, however, we are content to conclude that an increased live-weight gain and an increased efficiency of food utilization are obtained when sheep are fed 'a little and often' and that the reasons for this are as yet not understood.

In conclusion two aspects of this study that may have some application in (1) research and (2) practical agriculture should be mentioned.

(1) In digestibility studies where cut herbage is fed to animals under laboratory conditions it would seem to be important that they should be given the herbage a little at a time instead of, as is the usual practice, in two feeds at the most. These findings, and the fact that grazing animals do eat 'a little and often' (Tribe, 1949), would seem to indicate the necessity for this, although if our explanation, number (2) above, is the correct one, the effect obtained in this study under conditions of hay and concentrate feeding might not be reproducible when feeding herbage.

(2) It might be possible to apply this principle in the fattening of livestock. It should not be difficult to devise automatic methods for the frequent feeding of housed animals. The reduction in the amounts of food and time necessary for fattening and the consequent decrease in overhead costs would probably make this an economic proposition.

SUMMARY

1. Twenty-two Cheviot ewe hoggs gave greater body-weight gains and an increased efficiency of food utilization when offered their daily ration in eight small feeds than when given the same quantity and quality of food in one large feed.

2. The possible reasons for, and the possible applications of, these results are discussed.

We are indebted to Mr B. S. Walker for assistance in the management of the experimental animals, and to Mr J. L. Corbett for statistical advice.

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PROCEEDINGS
OF THE NUTRITION SOCIETY

SIXTY-NINTH SCIENTIFIC MEETING—THIRTY-SECOND
SCOTTISH MEETING
ROWETT RESEARCH INSTITUTE, BUCKSBURN, ABERDEENSHIR
21 SEPTEMBER 1951

'ALL FLESH IS GRASS'

Chairman: PROFESSOR T. L. BYWATER, *University of Aberdeen*

General Composition of Herbage

By S. J. WATSON, *Department of Agriculture, University of Edinburgh*

The most noticeable feature of any consideration of the chemical composition of herbage is that no one set of figures can possibly represent a particular plant or association of plants. This is an error into which the early investigators fell and it is common, even to-day, to see, for example, a single set of analytical figures quoted for a specific grass or clover. Most of the early figures of the agricultural chemist were based on single cuts of grasses and clovers, grown separately, and usually cut at a fairly advanced stage of growth.

This may not have been so great a mistake as would appear at first sight, because in some cases the investigators had in mind the use of the plant for hay and not for grazing. The samples were, therefore, cut at about the usual time for hay-making and took no account of the stage of growth of the plant at the time of cutting. In other investigations, however, there is a wide scatter in dates of cutting and the figures are of little value for comparative purposes since some grasses or clovers were cut while young, others at advanced stages of maturity.

Some of the Continental workers appreciated the importance of stage of growth, but the earliest reference in this country is the work of D. Wilson, later Sir David Wilson (1886, 1889), in the west of Scotland.

Main constituents

Before discussing the changes in composition which occur in herbage, it may be as well to refer briefly to the analytical groups into which the agricultural chemist has for many years divided the constituents of herbage. Though the divisions are rough, they have served their purpose tolerably well, so far as classification of the forages is concerned, and have enabled the feeding of farm animals to be conducted with a certain degree of accuracy. By their use the main principles of animal nutrition have been established and they have allowed of the formulation of feeding standards for farm animals, which have been used with every success. The usual methods of foodstuff analysis divide the sample into six main groups.

Moisture. In grassland herbage, moisture or water content is high and may vary from 65 to as much as 85 %; the younger the plant the higher will its moisture content be. The moisture content is one of the most important factors in fixing the value of a foodstuff since it determines the dry-matter intake. The ordinary silo in which young herbage is preserved by a fermentative process can be given as a simple example. The silage at the surface may contain 75 % moisture, and at the bottom up to 80 %. This does not seem a great difference, but the dry-matter intake of the animal which receives 100 lb. of silage would be 25 lb. when feeding on material from the top, and only 20 lb. when feeding on material from the bottom of the silo, a reduction of the order of 20 %, which is appreciable.

Similarly, since the plant tends to have a higher dry-matter content as it matures, any comparisons at different stages in the growth cycle should be made on a basis of the analysis of the dry matter of the herbage, and not of the fresh material.

Minerals. The ash or mineral matter is the second group, and as it does not usually form a large proportion of the dry matter it has received only lately the detailed attention that it deserves.

Nitrogen. The nitrogenous constituents are usually stated in terms of protein, using the factor of 6·25 to transform the value for total nitrogen into that for crude protein. This figure is sometimes further fractionated into 'true' protein, copper-precipitable nitrogen multiplied by 6·25, and a residue with the unwieldy title of non-protein nitrogenous material or sometimes, incorrectly, 'amides'. The crude protein, despite the shortcomings of the conversion factor, is a useful measure of the nitrogenous substances, since, except in the very early stages of growth, a high proportion of the nitrogen exists in complex forms that are readily precipitable by copper.

Fat. The fourth group, the ether extract, is often called crude fat and whilst it includes the true fats, it also includes a number of other constituents of which, for the agriculturist, the most important are undoubtedly the carotenoid pigments. They are the precursors of vitamin A, the accessory factor of greatest importance to the ruminant, the animal most suited to dealing with grassland herbage as its main or sole item of diet, though the horse may also be included in this category.

Fibre. The fifth group is the fibre, and this has a purely empirical value which does, however, give some clue to the relative ease of digestion of the sample and is, therefore, a useful figure.

Nitrogen-free extractives. The last and usually the largest group is that of the nitrogen-free extractives, and the value for it is obtained by difference and hence is a summation of all the errors inherent in the other methods of analysis. Its apparent advantage is enhanced by the fact that, being a difference figure, it means that all analyses of foodstuffs add up to 100 %, correct to as many places of decimals as the analyst may like to use. It is, however, fair to say that, despite their obvious shortcomings, the figures obtained have shown clearly the nature of the differences that exist between different components of the sward and between the different stages of growth within any one species in the herbage.

Changes in composition with growth

Wilson (1886) was the first to show clearly the changes in composition of herbage and to emphasize the fact that stage of growth is the most important factor. In his own words: 'It may be stated generally, that as the grass advances to maturity, the percentage of water in the green grass diminishes greatly. In the dry matter the percentage of albumenoids diminishes greatly; the ash and oil generally diminish, but less regularly and to a less extent. The woody fibre and extractive matter free from nitrogen, on the other hand, increase. The greater the amount of albumenoids they seem to be the more easily digested, so that the digestibility of albumenoids also decreases as the plant grows older.'

Then again: 'Compared with the difference of composition at different stages of growth, the differences between the composition of the various grasses cut at the same stage are small.' This latter point is of paramount importance, and indeed Wilson grouped all his analyses of grasses and of clovers, irrespective of species, under age groups.

The modern work of Fagan and his collaborators at Aberystwyth (Fagan & Jones, 1924; Fagan, 1929; Fagan & Milton, 1931) and of Woodman and his colleagues at Cambridge (Woodman, Blunt & Stewart, 1926, 1927; Woodman, Norman & Bee, 1928, 1929; Woodman, Norman & French, 1931; Woodman & Underwood, 1932) has amply confirmed and extended these findings. This and the work of other investigators has been summarized by Watson (1951). The most recent information is that of Waite & Sastry (1949), working at the Hannah Dairy Research Institute, in which a very complete study has been made of the composition of certain grasses during seasonal growth. The following figures, taken from the work of Fagan (1929), show clearly that the different grasses cut at a young stage are similar in composition. The values are for percentages in the dry matter:

Constituent	Cocksfoot	Yorkshire fog	Bent
Protein	24·3	23·9	18·9
Fibre	22·3	16·1	23·0

At this stage all three grasses, though of very different economic value because of their varying growth characteristics, are high in crude protein and low in fibre. Similar data are available for different clovers cut at equivalent states of maturity. At more advanced stages of growth the analytical values show the same trends.

Turning now to the differences in composition at different stages of growth in any one plant it is interesting to take Fagan's (1929) data for Italian rye grass, a quick-growing grass (Table 1).

The changes are exactly those which Wilson showed in his earlier work. In the ash the calcium is relatively steady throughout, but the phosphorus and potassium follow the nitrogen as it decreases with the age of the plant.

The simplest single figure to follow is the nitrogen, which can be determined with the greatest degree of accuracy and which is a useful guide to the general value of the plant.

The practical application of the changes can be shown most easily from the work of Shutt and his collaborators in Canada (Shutt, Hamilton & Selwyn, 1928, 1930, 1932). They used plots of meadow foxtail cut under different systems, and Table 2 shows the changes in protein content.

Table 1. *Composition of Italian rye grass expressed as percentages of dry matter at different stages of growth* (*Fagan*, 1929)

Constituent	At 2 weeks	At 6 weeks	At 10 weeks
Ether extract	3·75	2·42	2·10
Crude protein	18·54	12·12	6·90
'True' protein	13·32	7·80	5·47
Non-protein N × 6·25	5·52	4·32	1·43
Fibre	20·45	21·62	25·33
Nitrogen-free extractives	44·36	55·89	60·22
Ash	12·60	7·95	5·45
Calcium (CaO)	0·87	0·91	0·81
Phosphorus (P_2O_5)	0·88	0·45	0·37
Potassium (K_2O)	3·75	3·17	2·20

Table 2. *Crude-protein content of grassland herbage expressed as a percentage of dry matter in different seasons* (*Shutt, Hamilton & Selwyn*, 1928)

	1927	1928	1929
Cut weekly	21·20	28·95	29·15
Cut fortnightly	18·60	22·54	26·38
Cut every 3rd week	17·17	20·74	22·89
Cut as hay and aftermath	10·16	13·30	11·19

The increase in protein content in the first three plots in the 2nd and 3rd years was due to an incursion of wild white clover, due to the system of cutting, and shows that the leguminous plants show the same general trend, though they are normally higher in protein content than the grasses, especially under more lenient systems of cutting.

Effect of nitrogenous manure

It would seem that the stage of growth is, therefore, the dominant factor in deciding the protein content of grassland herbage and consequently in fixing the feeding value of the sward. Nitrogenous manuring may have an effect, and this is more marked at later stages of growth, apart altogether from its effect on yield of crop. Early American work was followed up in this country by Lewis (1939–40), who applied nitrogen to hay crops shortly before cutting (Table 3).

The protein content was raised by 1·5–3·0 percentage units by this treatment with little increase in yield of dry matter, most of the extra nitrogen being present in a complex form precipitable by copper. At earlier stages in growth, for silage cuts, there was still a response, though it was less marked according to later experiments.

The Aberystwyth work also gives a clue to a simple method of classification of grassland herbage; the proportion of leaf to stem determines to a considerable degree the protein content because of differences in composition of these two fractions. Fagan (1929) gives data (Table 4) for perennial rye grass that make this clear.

Table 3. *Effect on crude-protein content of hay of late applications of nitrogen (Lewis, 1939–40)*

Days before harvest ...	10		20	
Nitrogen applied (lb./acre)	17·4	34·7	17·4	34·7
Crude-protein content (percentage of dry matter) after application of:				
Sulphate of ammonia	10·77	11·84	10·63	11·14
Nitrate of soda	10·11	11·77	10·28	12·38
Nitrochalk	9·96	11·63	10·00	11·16
Mean	10·28	11·74	10·30	11·56
No nitrogen	8·69			

Table 4. *Composition of perennial rye grass, leaf and stem expressed as a percentage of dry matter (Fagan, 1929)*

	Young		Mature	
Constituent	Stem	Green leaf	Stem	Green leaf
Ether extract	2·45	2·45	3·30	3·65
Crude protein	15·50	24·75	10·37	17·68
'True' protein	11·31	20·37	8·75	13·68
Non-protein N × 6·25	4·19	4·38	1·62	4·00
Fibre	26·85	19·73	28·60	20·15
Nitrogen-free extractives	46·33	41·49	47·87	47·02
Ash	8·87	11·58	9·86	11·50

The leaf is richer in protein and ash than the stem, whereas the fibre and the nitrogen-free extractives are highest in the stem. In addition, the proportion of leaf to stem alters with age; the most complete study of this change is that of Waite & Sastry (1949), who show with timothy grass a ratio of 2·5 parts of leaf to 1 of stem in the early part of the season, falling to less than 0·5 part of leaf to 1 of stem as the plant approaches maturity.

Feeding value

The leafiness of pasture grass will, therefore, give a simple and relatively accurate method of forecasting its feeding value. This is shown in Table 5 taken from the work of Watson (1949).

Table 5. *Composition and feeding value of pasture grass (Watson, 1949)*

Stage of growth	Dry matter	Crude protein	Ether extract	N-free extractives	Fibre	Ash	Starch equivalent (S.E.)	Protein equivalent (P.E.)	Ra S./P.
Very leafy	18	4·0	0·6	7·5	3·6	2·3	10·8	2·8	
Leafy	19	3·3	0·5	8·5	4·5	2·2	11·3	2·2	
Early flowering stage, little stem	21	3·0	0·7	9·8	5·4	2·1	12·2	1·9	
Flowering stage, stemmy	23	2·4	0·5	11·7	6·2	2·2	12·7	1·5	
Full flower, seed stage	25	2·1	0·6	13·1	7·4	1·8	12·8	1·2	1

These values are based on actual digestibility trials and show in general the changes that have already been described. It should be realized that the figures are for the fresh material and if they are compared on a dry-matter basis, as they should be, the changes will be accentuated. This shows most clearly for the energy value or starch equivalent of the herbage which seems to increase with age, but on a dry-matter basis the values would be 59·9, 58·9, 58·5, 57·4 and 50·4 for the samples from very leafy to full flower respectively.

Energy and protein value. It is evident that the value of the herbage as regards its energy and protein value is correlated with its age, which in turn is related to the leafiness of the sward. In considering any particular pasture it is necessary to bear in mind its age, and a useful practical guide to its value will be the proportion of leaf to stem. It is not necessary to take into account the actual components of the sward if it is a mixture of grasses with a proportion of clover of the prostrate type. If, however, the herbage is predominantly leguminous, a similar set of figures is available and is again based upon the age of the sward, irrespective of the actual plants of which it is composed.

Minerals. Though the main basis of evaluation of herbage is its content of energy and of digestible protein, other factors must be taken into consideration. The mineral matter is most important, but it will have been evident from the figures already shown that the ash content tends to vary with the nitrogen, this being especially true of the phosphorus and potassium.

Further confirmation of this comes from the work of Elliot, Orr & Wood (1925), who examined typical pastures in England and Scotland. Their classification gave cultivated pastures an average value of 2·83 % nitrogen, 1·00 % calcium and 0·74 % phosphorus (stated as N, CaO and P_2O_5). Good natural pastures gave 2·50, 0·65 and 0·67 % respectively, poor natural pastures (eaten) 2·54, 0·56 and 0·60 %, and poor uneaten, natural pastures contained only 1·82 % nitrogen, 0·30 % calcium stated as the oxide and 0·37 % of phosphorus, stated as phosphorus pentoxide.

The soil status will have a marked effect on the mineral content of the herbage, and in cases of deficiency the stage of growth will afford no clue to the amounts of the various constituents present. This will be most marked in the case of the trace elements where the stage of growth and the relative leafiness have no relation whatsoever to the amounts of the different elements present (Watson, 1951).

Vitamins. As far as vitamins are concerned, vitamin A is the most important factor, and the relation of its precursor, carotene, to the stage of growth is of interest. Waite & Sastry (1949) have made a full study of this and show that the carotene content of the grasses is closely related to the nitrogen content, so that here again relative leafiness is a valuable guide to the amounts of carotene present. This is true of the fresh grass, but when it is cut for haymaking, artificial drying, or ensilage there may be a loss of carotene and this will vary with the time of exposure and with climatic conditions immediately after cutting. These workers found, for example, that in hot sunny weather as much as 50 % could be lost in 24 h and in cases of extreme exposure 80 % could disappear.

Other factors. In conclusion, it should be remembered that other factors must be taken into account in interpreting the feeding value of pasture. The practical man will claim that, despite the analysis, grass in autumn has not the productive value of spring grass. Morris, Wright & Fowler (1936), at the Hannah Dairy Research Institute, suggested that it was due to variations in the biological value of the protein, and more recently the work of Legg, Curnow & Simpson (1950) has focused attention on the oestrogens in grass which they show tend to be higher in spring grass.

Then again there is the marked laxative effect of young grass, particularly for cattle that have just been turned out, a factor which markedly affects its value at that time. Finally, we have troubles such as blowing or bloat in cattle on grass at certain periods of the year when the animals are unable to eliminate the rapidly formed gas from the rumen, and the work of Ferguson (1948) has shown the presence of a compound which affects muscle activity and is present in legumes at certain seasons.

A good deal more information is still needed before a final assessment can be made of the feeding value of grassland, and the animal itself is the only final and accurate yardstick. It is, however, possible to forecast the value for most purposes more accurately to-day than before.

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The Utilization of Herbage Protein by Animals

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Between 80 and 90 % of the nitrogen of herbage is, beyond question, protein. The remainder, the so-called 'non-protein nitrogen', consists mostly of free amino-acids, but also includes nucleic acids, purine and pyrimidine bases, choline and betaines, alkaloids, peptides, ammonia, urea, nitrate and other compounds. Chibnall (1939) and Lugg (1949) have well summarized much of the chemical information available. Most of the protein seems to be in the chloroplasts; the substances of low molecular weight behave as if in solution in the tissue juices.

The usefulness of protein to such animals as the rat, the chicken, the pig and man is understood nowadays to depend chiefly on amino-acid composition and particularly on the content of about ten so-called 'essential' amino-acids. These are not well represented among the free amino-acids of the tissues of the green plant, where the 'non-essential' glutamic and aspartic acids and their amides, glutamine and asparagine, with alanine, serine and γ-aminobutyric acid tend to predominate, much as in the blood and tissue juices of animals. However, the proportions of amino-acids in the true protein of the leaf are much more favourable to the requirements of these animals, vary very little with season or species, and put the leaf proteins into the category 'first class' (although there is a tendency for methionine to be low, as in so many other proteins). Thus for these animals leaf protein is more valuable than most other types of plant protein. Given suitable maceration to render it accessible, leaf protein is well hydrolysed by the intestinal proteases. Its drawback as a main source of protein is the large quantity of indigestible material, mainly carbohydrates, that accompanies it, and this is the main factor limiting its consumption by these animals.

Some animals, however, live mostly on leaves. They include such domestic animals as the horse, the rabbit, the goose and, most important, the ruminating cow, sheep and goat. In all of them the intestines are specially adapted to give space and time for extensive microbial fermentation of the leaf carbohydrates. In the non-ruminants mentioned this adaptation is in the caecum and colon, the stomach and small intestine being little modified. In the ruminants there is great modification of the intestinal tract above the stomach, the food undergoing microbial fermentation in the rumen immediately on ingestion. Too little is known of the digestive physiology of the non-ruminant herbivores, and I will therefore only make two points about them. First that digestion and absorption of leaf protein may be fairly complete in the upper alimentary tract, and their protein economy therefore much the same as that of non-herbivores. Presumably the protein of the micro-organisms of their large intestines is mostly lost. The source of nitrogen for growth of these organisms may well be excess nitrogen from the animal's metabolic pool that would otherwise be excreted, entering the gut as compounds of low molecular weight from the blood; it would thus represent little serious nitrogen loss to the animal; the coprophagy of the rabbit probably turns this loss to gain. My second point is that the digestive physiology of these animals ought to be studied in parallel with that of the ruminant since, regardless of direct economic motives, such knowledge may well hasten our understanding of ruminant digestion and perhaps of other means of making use of herbages. The economic stakes are to be calculated in millions of pounds.

Although the protein metabolism of ruminants has been a controversial subject for half a century, it is possible to give a non-controversial statement in qualitative and general terms of what happens to nitrogen compounds in the rumen.

Protein of the food is in part broken down by micro-organisms and in part passes on unchanged to the abomasum (or true stomach); part of what is broken down by the micro-organisms is assimilated by them and built into microbial protein which likewise passes on to the abomasum. Another part is converted to ammonia and is absorbed by the animal directly from the rumen. Urea and other nitrogenous compounds of low molecular weight in the food, in the saliva or entering the rumen from the animal's blood may undergo the same treatments by the micro-organisms. It is also very generally assumed that at least a fair proportion of the protein digested by the animal in the abomasum and small intestine is microbial protein, and it has been shown experimentally that the protein of mixed rumen micro-organisms is a high-grade, well-balanced protein for rats (McNaught, Smith, Henry & Kon, 1950). The fact that ruminants show none of the signs of amino-acid imbalance on being fed exclusively on proteins that are ill-balanced for rats, chickens and men is therefore taken to be explained by the contribution of microbial protein, and the requirements for absorbed amino-acids are assumed to be not dissimilar to those of the animals whose requirements have been studied in detail.

Beyond this point nearly everything is controversial. Because the mixture of amino-acids absorbed by the ruminant is a well-balanced one, it does not follow that the overall utilization of protein is always efficient. Two opposing tendencies can be seen at work: (*a*) non-protein nitrogen, poor in 'essential' amino-acids, entering the rumen is ennobled to microbial protein; (*b*) food protein of good amino-acid composition is attacked in the rumen and converted into ammonia which is to a great extent absorbed (McDonald, 1948*a*) and excreted as urea.

The important thing is to ascertain, in particular instances, whether processes of type (*a*) or of type (*b*) predominate. This has very rarely been done unequivocally, and it is worth pointing out here that the conventional 'digestibility' studies, traditional to agricultural chemistry, are ill-adapted for the purpose; to know the net absorption of nitrogen from the intestine provides no evidence whether it has entered the animal as ammonia or as essential amino-acids. At this stage of development of the subject practical experiments on growth, milk yield and so forth or else thoroughgoing nitrogen-balance experiments, in which urinary nitrogen is measured, seem preferable to 'digestibility' experiments.

It seems conclusively established that supplementation of certain diets with urea can lead to good use being made of this supplementary nitrogen. This is known to depend very much on the type and amount of carbohydrate simultaneously fed. The best results have been with beef cattle, and fewer favourable experiences with dairy cattle (which require a higher protein concentration in the diet) have been recorded. It is perhaps significant that these favourable experiences have mainly been in the U.S.A., where maize plays a much larger part in cattle feeding than it does in northern Europe. McDonald (1948*b*, *c*, 1952) has studied the rates at which different proteins were converted to ammonia in the rumen of the sheep, and found zein, the chief protein of maize, to be converted very slowly indeed. He was able, by making use of its unusual solubility in aqueous alcohol and of its low lysine content, to study quantitatively the disappearance of zein and the simultaneous formation of lysine-rich microbial protein in the rumen. It would help if similar specific tests were available for studying the fate of other proteins. Experiments here have confirmed that casein is very rapidly deaminated in the rumen, and is consequently of little value as a protein supplement when administered to sheep unless the rumen is by-passed and the casein is given directly into the duodenum (Cuthbertson & Chalmers, 1950). It has also been possible to correlate the greater value of herring-meal supplements over casein supplements for sheep with the lower evolution of ammonia that is observed from the herring meal (Chalmers & Synge, 1950, and unpublished work).

From all this it is evident that quite other factors than amino-acid composition determine the value of protein or non-protein nitrogen to a ruminant animal: the amount and nature of the carbohydrate that is being simultaneously fermented, the solubility of the protein and its susceptibility to the microbial proteases are obviously among the important factors.

An examination of the utilization of herbage protein from this angle reveals a host of problems, as well as the futility of attacking them without paying simultaneous attention to the fate of the carbohydrates. I cannot do more than name a few of the problems that seem relevant at the present time. Heavy nitrogenous manuring of pastures is essential for high yields, and this at the same time raises the protein content of the grass and lowers the content of soluble carbohydrate. The soluble-carbohydrate content also fluctuates greatly with season, and it seems possible that these changes, rather than changes in protein content, may be responsible for some of the differences that are said to exist between the value of spring and autumn grass. Again, it is quite possible that in modern highly fertilized pastures protein content has been pushed altogether too high for efficient utilization, and that dairy cattle should have some supplementary feeding with carbohydrate all the year round. The special hazard of high nitrate content in the herbage should be noted in view of the danger of nitrite formation in the rumen (Lewis, 1951). Finally, I think a big mistake has been made in assessing conservation methods solely on the conservation of 'digestible crude protein' without reference to practical results. This sort of approach has done much to lower the reputation of science in the eyes of the practical man. The question of hay versus silage is not settled by quoting figures for digestible crude protein. The making of silage results in conversion of much of the protein of grass into soluble nitrogenous compounds of lower molecular weight and in fermenting away much of the soluble carbohydrate present. The consumption of silage may thus lead to a predominance of deaminative reactions over protein synthesis in the rumen. With hay, this predominance cannot be so great. So far there has been too much propaganda and too little experiment in matters of grass conservation.

There are no reasonable evolutionary grounds for regarding the ruminant as well adapted for digesting and assimilating protein under modern dairy conditions. The primary adaptation of the ruminant is clearly towards the use of carbohydrates not available to other animals. Under wild conditions at some seasons (coinciding with parturition and lactation, when the physiological demand for protein is greatest) abundant protein is available in the herbage and the animal can afford even to waste it. At other times, when the animal has to live on a few old sticks and straws, a very efficient mechanism for recovering protein from the waste nitrogen compounds of its own body fluids comes into play, and this has probably been the aspect of protein digestion in the ruminant that has undergone the greatest evolutionary adaptation.

If I have succeeded in showing how complicated the problems are and what a little way we have advanced towards understanding them, I shall be quite satisfied. It will require a much greater knowledge of the rumen micro-organisms, of the chemistry of herbage and of the biochemistry and physiology of the fermentations and absorptions that are proceeding before genuinely scientific recommendations can be made. It is sheer presumption to recommend changes in farm practice on the basis of a few Kjeldahl figures.

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The Carbohydrate Constituents of Herbage

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Although an immense amount of routine analytical work has been carried out on forage crops in the past, we are still ignorant, in the main, of what is measured under such headings as crude fibre, total carbohydrate and so on. With the advent of new techniques such as chromatography and desalting by ion-exchange and electrophoretic methods, work is now in progress to fill in these gaps in our knowledge. It is still too early to do more than indicate the probable nature of the carbohydrate components, much less to evaluate them quantitatively, and it may be many years before a complete picture can be drawn. It is perhaps appropriate at this point to emphasize the need for the examination of pure species grown and harvested under controlled conditions so that the seasonal variations of the constituents, for example the fructosans, may be followed.

For convenience we may divide the carbohydrates of herbage into those that play a part in the structure of the plant, and the non-structural components such as the free sugars and oligosaccharides and the reserve polysaccharides.

Structural polysaccharides

Cellulose

The most important structural component from the quantitative standpoint is, of course, cellulose, and although no studies of the fine structure of cellulose specimens obtained from herbage appear to have been carried out, it is reasonable to suppose that there is no essential difference between cotton (or wood) cellulose and the cellulose of herbage. Indirect evidence in favour of this assumption is afforded by the fact that celluloses isolated from such dissimilar sources as the cell-wall of the pear (Hirst, Isherwood, Jermyn & Jones, 1949) and of marine algae (Percival & Ross, 1949) are fundamentally the same as cotton cellulose, being composed of long chains of 1 : 4-linked β-D-glucopyranose residues. One would hesitate to affirm, however, that the molecular sizes or chain lengths of all plant celluloses are necessarily the same, and in fact the marine algal cellulose appears to have a shorter chain than standard cotton cellulose.

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* Died on 27 September 1951.

Pentosans

Xylan. There is abundant evidence that a xylan (or xylans) occurs in herbage because considerable amounts of xylose are produced on the hydrolysis of certain hemicellulose fractions isolated from cocksfoot grass (Buston, 1934), and the amount is possibly of the order of 5–10 %. In no instance, however, has a homogeneous xylan been isolated and its chemical architecture determined. This point is of some importance because it is now becoming clear that several xylans exist. Thus, the one isolated from esparto grass and which has been the most intensively studied consists of 1 : 4-linked β-D-xylopyranose units contained in a singly branched or forked molecule, the branch being formed through a hydroxyl group on $C_{(3)}$ (Chanda, Hirst, Jones & Percival, 1950). The molecule contains some eighty xylose units. The xylan isolated from pear cell-wall (Chanda, Hirst & Percival, 1951) also possesses a forked structure but contains also a D-glucuronic-acid residue as a terminal group, and the molecule is somewhat larger (about 110 units) than that of esparto xylan.

Finally, a xylan has been isolated from the red seaweed *Rhodymenia palmata* which differs from both the previous examples in containing chains of xylopyranose residues linked by both 1 : 4- and 1 : 3-linkages (Percival & Chanda, 1950). With these different examples before us, it would be unwise to speculate too freely on the constitution of herbage xylans, although one would expect the general picture to be similar to that of the first two examples mentioned.

Arabans. No pure arabans have yet been isolated from herbage, although there is evidence for their presence in cocksfoot grass (Buston, 1934) and in clover (Laidlaw & Reid, 1952). Arabans are always associated with pectin (Hirst, 1949), and the arabans isolated from such materials as the groundnut (*Arachis hypogaea*) or sugar beet are known to have highly branched structures. Great difficulty was experienced in isolating the araban specimens for these structural determinations even from sources comparatively rich in the polysaccharide, so that it will necessarily be a difficult matter to determine whether the arabans of herbage conform to the same general pattern.

Galactan

Since the hydrolysis of the holocellulose of pasture grass and of clover gives indications of the presence of galactose it is presumed that a galactan is present. Together with araban a galactan is always found in association with pectin (Hirst, 1942), and evidence has been presented that the polysaccharide in question is composed of D-galactose residues united by β-linkages through the 1 : 4-positions. So far the quantity present in herbage is unknown, and it may be a difficult matter to determine the precise constitution.

Pectin

Although pectic acid undoubtedly exists in herbage (Nanji & Norman, 1928), especially when young, it is not a major constituent and little work appears to have been carried out on the examination of herbage pectins. Apart from its isolation as calcium pectate, the presence of pectin may be inferred from the fact that arabans and galactans are present and that D-galacturonic acid has been identified on hydrolysis (Buston, 1934). It is of interest to note that the amount of pectic acid present as the methyl ester is greater in grass than in hay, although in both instances the pectic acid appears to be principally in the form of mineral pectates (Buston, 1935). Buston presents evidence that pectin is not a precursor of lignin, and his conclusion, that the hemicelluloses in green unlignified leaves are mainly galactans and arabans, and in older lignified tissues are glucosans and xylans, is of considerable interest, especially in regard to legumes.

Glucosans

There is some evidence that glucosans may be present in herbage, because after the extraction of clover and lucerne with 80 % ethanol to remove sugars and oligosaccharides the aqueous extract can be shown to give some glucose on hydrolysis as well as xylose, arabinose and galactose (Laidlaw & Reid, 1952). It may be recalled that a water-soluble glucosan was isolated from barley roots (Hassid, 1939) in which it appeared that the glucose residues were united by 1 : 6-linkages. It is necessary to keep an open mind as to the source of the glucose in the hydrolysates mentioned above, but in the apparently complete absence of starch in the foliage of herbage the possibility cannot be excluded that a similar glucosan may be present in small amounts in forage plants.

Non-structural carbohydrates

Monosaccharides

Glucose and fructose appear, in small quantities, as herbage constituents, and are subject to seasonal variations. In general the fructose content is between 1 and 2 % and the glucose of the order of 1 % (de Man & de Heus, 1949; Laidlaw & Reid, 1952). Preliminary investigations of the seasonal variation have been carried out in Edinburgh and a more systematic investigation is now in progress.

Oligosaccharides

Sucrose. Sucrose is, quantitatively, the most important sugar in herbage, and the amount is surprisingly large, values of 5–6 % having been recorded (de Man & de Heus, 1949; Laidlaw & Reid, 1952).

Melibiose and raffinose. There is evidence from chromatographic examination that small amounts of the reducing disaccharide melibiose (6-[α-D-galactosido]-D-glucopyranose) occur in grass extracts (Laidlaw & Reid, 1952). The presence of this sugar is not unexpected since the trisaccharide raffinose also appears to be present, which by the loss of a fructose residue can give melibiose.

Stachyose. The tetrasaccharide stachyose also appears to be present in the 80 % ethanol extracts of grass. It may be recalled that both raffinose and stachyose have been found in apples, plums and pears (Bradfield & Flood, 1950). Raffinose contains, of course, a sucrose residue and on hydrolysis gives sucrose and galactose or fructose and melibiose, or glucose, fructose and galactose depending on the conditions employed. Stachyose also on hydrolysis gives glucose, fructose and two molecules of galactose, and although the presence of a sucrose residue in this tetrasaccharide has not been definitely proved, and indeed the precise structure still requires final elucidation, the possible interdependence of all the non-structural components mentioned so far needs little emphasis. The same is true indeed of the highly important fructosan fraction which is the remaining topic for discussion.

Fructosan

From at least two standpoints the fructosan component of the herbage carbohydrates is of the highest importance. In the first place, as far as the metabolism of the plant is concerned it is the reserve carbohydrate, and in the second, I believe that it will be found to play an important part in the making of good ensilage, because, since the fructosans are all constructed from fructofuranose residues, they are by far the most easily hydrolysed of all the polysaccharides.

The experiments of de Man & de Heus (1949) on rye grass show an apparent variation in fructosan content from 5 % when cut on 23 April to 17·6 % when cut on 7 June. In the preliminary experiments in Edinburgh the values determined by chromatographic methods were 6·8 % (3 May) and 12·1 % (6 June) for perennial rye grass and 2 % and 4·9 % for cocksfoot cut on the same dates (Laidlaw & Reid, 1952). It is of great interest to note that no fructosan has been detected in clover or lucerne.

Reference may be made to an important review by Archbold (1940) which, although primarily concerned with the fructosans of such plants as barley and wheat, is of general interest. Several workers have analysed the leaves, stems and ears of wheat, barley and rye at various stages during growth (Colin & Belval, 1922; Belval, 1924*a–c*; Barnell, 1938). Fructosan appeared in the leaf sheaths of wheat in quantities amounting to 0·5 % of the fresh weight on 19 May, increasing to 2·6 % on 11 June. Fructosans were absent in the stems at first, but as the spikes emerged the polysaccharide began to accumulate and reached a maximum at the heading period, reaching a value of 5 % in wheat stalks. The fructosan in the ears, as estimated as soon as it was possible to dissect them, amounted to 6–8 % and this fell continuously during development. Later workers (Archbold, 1938; Barnell, 1938) showed that fructosan was also present in the leaves of barley, although it is thought that the fructosans are produced not in the leaves but in the stems. For wheat and barley it is suggested that the fructosans may be, in part, the precursors of starch (Archbold, 1940). In any case it is clear that the fructosans are stored only temporarily in the stems during a relatively short period when the leaves deliver more sugar than is immediately necessary for the growth of the ear. Although this is not the place to discuss the merits or otherwise of these views, it is clear that from the point of view of plant physiology the measurement of distribution of fructosans in the various parts of the plant is very important.

The chemical constitution of fructosans. Although many specimens of fructosans have been isolated and named (Archbold, 1940) detailed analysis has been confined to but a few, H. H. Schlubach having been the foremost worker in this field.

With grass fructosans the first structural analysis was carried out in Birmingham

(Challinor, Haworth & Hirst, 1934*a*) on a product isolated from rough-stalked meadow grass (*Poa trivialis*). This substance was shown to have a structure represented by a chain of D-fructofuranose units linked through $C_{(2)}$ and $C_{(6)}$, and was quite different from the root-reserve fructosan inulin in which the same units are linked through $C_{(2)}$ and $C_{(1)}$.

The grass fructosan was in fact apparently identical as regards its main structural features, although not necessarily in its molecular size, with the product, levan, which is obtained by the action on sucrose of the micro-organism *Bacillus mesentericus* (Harrison, Tarr & Hibbert, 1930; Hibbert, Tipson & Brauns, 1931; Challinor, Haworth & Hirst, 1934*b*). The soluble fructosan from barley leaves already mentioned (Archbold & Barter, 1935) was also found to contain the same fundamental 2 : 6-linkage (Haworth, Hirst & Lyne, 1937), and this linkage is found also in the fructosans isolated from other grasses, e.g. the so-called phlein from timothy (*Phleum pratense*) (Schlubach & Sinh, 1940), the fructosan secalin from unripe rye (Schlubach & Bandmann, 1939), and pyrosin from the stalks of wheat (Schlubach & Huchting, 1949). So frequently has the 2 : 6-type of linkage been established in fructosans isolated from stems and leaves that it has been proposed (Schlubach & Sinh, 1940) that two main groups of fructosans, called the phlein type and the inulin type, should be considered to exist.

More recent examinations of grass fructosans have been made and the 2 : 6-linkage has been established for all of them. Bell & Palmer (1949) have concluded that the fructosans of Italian rye grass and leafy cocksfoot contain about fourteen fructofuranose units in a chain, although the molecular weights determined by physical methods (ultracentrifuge) show molecules containing about thirty units, which implies branched-chain structures. The fructosan from perennial rye grass (Laidlaw & Reid, 1951) appears to have a chain length of twenty-five to thirty fructose units, and the same is true of a specimen from leafy cocksfoot (Percival & Telfer, 1951). In these instances the results do not suggest that the molecules are branched, and it is clear that further work is necessary to determine whether, by the different methods of isolation adopted by the two schools, different fructosan fractions are obtained.

In the suggested constitution for the perennial rye grass fructosan the molecule is terminated by a sucrose residue. Such a type of end-group was first postulated for inulin (Hirst, McGilvray & Percival, 1950) to account for the results of experiments by methylation and periodate oxidation, and a similar terminal grouping has been postulated for the fructosan triticin of couch-grass rhizomes (Arni & Percival, 1951). This brings me to the point that it is very difficult, if not impossible, to isolate any fructosan that does not yield a small quantity of glucose on hydrolysis, and by the autohydrolysis in water of several fructosans we have found it possible to demonstrate the presence of sucrose. The possibility exists, therefore, that the natural fructosans are built up from sucrose by the attachment of fructofuranose units to either $C_{(1)}$ to give the inulin type, or to $C_{(6)}$ to give the grass-fructosan type of structure. Incidentally it may be pointed out that the stereochemical nature of the two structures is quite different, the former being composed of fructofuranose units arranged one above the other like a pile of plates or the leaves of a book, and the other as an elongated molecule, facts which surely explain the relative insolubility of inulin in water as compared with the soluble and readily diffusible phlein-type fructosans.

It has been proposed (Palmer, 1951) that the molecular size of a fructosan can be calculated simply by estimating the glucose content on the assumption that one glucose residue is present in a molecule, and good agreement is obtained in most cases.

In connexion with the postulated sucrose (or sucrose-type) end-group, the work of Bacon & Edelman (1951) in Sheffield on the carbohydrates of the Jerusalem artichoke should be mentioned. These workers have demonstrated that at least seven non-reducing components are present with R_F values on the paper chromatogram ranging from that of sucrose to zero, the latter presumably corresponding to inulin. These substances are present in the stems, tubers and roots of the Jerusalem artichoke and in the underground organs of seven other species of Compositae, and when the implications of these results are fully understood there is little doubt that our knowledge of the synthesis and breakdown of fructosans in the plant will be considerably advanced.

In conclusion, although it does not come strictly into the category of herbage, one may be permitted to mention the interesting fructosan triticin isolated from the rhizomes of twitch or couch grass (*Triticum repens* L.). In this polysaccharide the fructofuranose units are linked by both 1, 2- and 2, 6-linkages in equal proportions, and the fructosan is thus a hybrid between the phlein type characteristic of grasses and the inulin type found in tubers such as the dahlia and artichoke (Arni & Percival, 1951). The molecule again appears to contain about thirty units and a terminal sucrose end-group. Whether such a fructosan occurs in stems and leaves is, possibly, doubtful, but its very existence as well as that of others of similarly anomalous structure such as irisin from the wild iris (*Iris pseudoacorus*) makes it even more desirable that a thorough study should be made of the fructosans from sources other than those already mentioned.

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The Utilization of the Minerals, Vitamins and other Constituents of Grass

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Animals that spend their lives on pastures must receive from the herbage all the essential nutrients they require for growth and reproduction. The utilization of the proteins and carbohydrates of grass has already been discussed (Synge, 1952; Percival, 1952), and it now remains to examine the utilization of minerals and vitamins. At the same time, however, the presence of toxic factors in grass and the nutritional hazards which beset the grazing animal must be considered.

Before the adequacy of grasses and clovers in terms of mineral and vitamin content can be assessed, some approximate idea of the requirements of farm livestock for these entities must be obtained. In this respect recommended dietary allowances (Guilbert & Loosli, 1949, 1951) may not provide an adequate assessment, since such requirements of minerals and vitamins tend to be overestimates and include large margins of safety. Minimum requirements (Mitchell & McClure, 1937), expressed as percentages of the dry matter of the ration, should give an indication of the lower limits which are permissible in the composition of the diet. A pasture or grass containing less than this quantity can then be judged to be deficient. This does not mean that deficiency symptoms in their classical sense will arise on such pasture. Animals have considerable abilities to adapt themselves to suboptimal nutrition. These adaptive processes, however, inevitably involve slight declines in growth rate, milk yield, egg production and reproductive performance.

High rates of growth and production not only entail higher requirements per head of stock, but higher concentrations of most nutrients in the dietary dry matter. Thus to produce one egg a day the hen requires 3 % calcium in her diet whereas to produce none she requires only one-tenth this amount. Similarly, the mature cow requires as an absolute minimum 0·17 % phosphorus in her diet if she produces no milk and is not pregnant. She requires half as much again to produce 3 gal. milk a day, and almost double this quantity when lactating during the terminal phases of pregnancy.

Table 1 summarizes data on minimum requirements of Ca and P for farm livestock. Methods and data used for this purpose are given in the footnote to Tables 1 and 2.

Table 1. *Minimal calcium and phosphorus requirements of growing and producing animals expressed as a percentage of dry matter ingested**†

	Growing		Producing (gestation, lactation, egg production)	
Species	Ca	P	Ca	P
Horse	0·3	0·25	?	?
Sheep	0·15–0·2	0·15–0·2	0·2	0·2
Cow	0·2–0·3	0·2–0·3	0·3	0·3
Pig	0·3	0·3	0·5	0·4
Hen	0·8	0·4	3·0	0·5

* Requirements depend on the rate of growth and of milk and egg production, but the values quoted do allow for high levels of production. The values for growing animals do not apply to the very young suckling when they approach 1 % of the dry matter for Ca and 0·6 % for P.

† The food intakes of animals of different species in terms of dietary dry matter were taken from Guilbert & Loosli (1949, 1951). The Ca and P requirements are essentially those calculated by Mitchell & McClure (1937) and reviewed and amended by Mitchell (1947). They agree very well with experimental observations made with rations containing minimal quantities of Ca and P. Thus, minimal P requirements have been assessed from feeding experiments with cattle to be more than 0·13 and less than 0·26 % of the dry matter of the ration (Henderson & Weakley, 1930; Reed & Huffman, 1930). In general, the lower limits of Ca and P requirements have been taken. The copper and cobalt requirements are based on the data assembled and reviewed by Russell (1944). The manganese requirement derives from studies of Lyons (1939) and of Gutowska & Parkhurst (1942). No data on the requirement of cattle or sheep are available. Vitamin A requirements are based on a requirement of 25–30 μg β-carotene/kg body-weight (Guilbert, Howell & Hart, 1940) together with data on food intake. Vitamin D requirements were recalculated from the data of Guilbert & Loosli (1951) and are recommended allowances rather than minimal quantities. Requirements of aneurin were based on a consideration of the data of Arnold & Elvehjem (1938) and Van Etten, Ellis & Madsen (1940). Cowgill's studies (Cowgill & Palmieri, 1933) have shown that the aneurin requirement is determined by food consumption rather than metabolic rate. Requirements of the remainder of the vitamins of the B complex have also been referred to food intake, and the values given are those computed by Mitchell (1950). Since the animal requires most minor elements and B-complex vitamins to furnish part or all of the prosthetic groups of enzymes or to activate enzymic processes involved in the metabolism of dietary energy and protein it is logical to refer requirements for these nutrients to the quantity of food ingested rather than to body size or function. This step greatly facilitates the appraisal of dietaries.

The generalization of the requirements of individual species has been made in such a way as to avoid possible malnutrition of one species. Thus, with respect to Ca and P, as shown in Table 1, the horse has the highest minimal requirement of the three herbivora listed. These values for the horse have been taken as minimal requirements for herbivorous livestock, since their use as a criterion of adequacy then allows for a full utilization of a pasture or dietary regimen by all herbivorous species.

Table 2. *Approximate minimal requirements of essential nutrients for herbivora and for pigs and poultry, expressed in relation to the dry matter ingested. The requirements for high levels of egg and milk production may not be adequately covered**

	Species	
Nutrient	Herbivora	Pigs and poultry
Calcium (%)	0·3	0·8
Phosphorus (%)	0·25	0·5
Copper (mg/100 g)	0·3	?
Cobalt (mg/100 g)	0·01	Not required†
Manganese (mg/100 g)	?	4
Carotene (mg/100 g)	1	0·5
Vitamin D (i.u./100 g)	45	45
Aneurin (mg/100 g)	Not required	0·1
Riboflavin (mg/100 g)	Not required	0·15
Nicotinic acid (mg/100 g)	Not required	1·00
Pantothenic acid (mg/100 g)	Not required	0·5

* See footnote to Table 1.

† Required as a component of vitamin B_{12}.

Except for the hen, Ca requirements may be placed at not less than 0·3 % of the dry matter of the ration and P requirements at not less than 0·25 %. These figures are only rough approximations. They do not take into account the high requirement of the very young animal. The cow or ewe, however, will provide milk containing up to 1·5 % Ca and 1·4 % P in the dry matter to meet this requirement of the suckling. The requirement of the pig for very rapid growth is higher than the minimum listed, and so we can perhaps pool pigs and poultry with requirements approximately double those of herbivora.

Table 3. *Limitations of grasses, clovers and pastures in meeting minimal calcium and phosphorus requirements*

	Ca (percentage of dry matter)	P (percentage of dry matter)
Minimal requirements:		
Herbivora	0·3	0·25
Pigs and poultry	0·8	0·5
Content in:		
Individual plant species grown on good soils:		
Clovers*	1·0–2·0	0·25–0·60
Grasses	0·3–0·4	0·10–0·30
'Herbs'	Up to 3·0	0·25–0·40
Moorland plants (e.g. *Nardus, Molinia, Vaccinium*)†	**0·09–0·5**	**0·08–0·40**
Pastures (mixed species):		
Cultivated pasture‡	0·78	0·34
County Dublin best pasture§	0·56	0·52
Hannah Institute fertilized pasture‖	0·90	0·40
Good Scots hill pasture‡	0·39	0·26
Aphosphorosis area, South Africa¶	0·35	**0·08**
Aphosphorosis area, Offaly§	0·84	0·13
'Croitich' area, Scotland‡	**0·15**	**0·13**
'Knochenbrüchigkeit' area, Austria**	0·49	**0·10**

Bold figures indicate values lower than the estimates of minimal requirements of herbivora.

* Fagan (1931); Mitchell & McClure (1937); Thomas & Thompson (1948); Watson (1939); Schneider (1947).

† Thomas, Escritt & Trinder (1945); Thomas & Trinder (1947).

‡ Orr (1929).

§ Sheehy, O'Donovan, Day & Curran (1948).

‖ Holmes (1949).

¶ Theiler, Green & Viljoen (1915).

** Roloff (1866).

Similar tabulations have been made for other nutrient requirements using what scanty information is available in the literature, and generalizations regarding requirements of herbivora and of pigs and poultry have again been made; Table 2 summarizes what may be taken as lower approximate limits for the essential nutrients that grass should provide. Some entries in the table need explanation. The carotene requirement of an animal is directly in proportion to body-weight and is approximately 30 μg/kg body-weight (Guilbert *et al.* 1940), whereas food capacity tends to be proportionate to surface area. The requirements of small animals for carotene are therefore smaller per unit of food than those of large animals. Similarly, the requirements for the vitamin B complex are, broadly speaking, proportional to the non-fat calories metabolized, and the minimal requirements given are based not only on experimental results with pigs and poultry but also on trials with rats, dogs and man.

Calcium and phosphorus. With these base-lines of minimal requirement established, we can now examine pastures and individual species. Table 3 summarizes information on the Ca and P content of herbage from different areas. The table shows the superiority of clovers over grasses as sources of Ca and P, as well as the value of certain so-called herbs. It also emphasizes the inadequacy of the poor moorland plants of highland grazings. Some pastures listed are high-quality pastures and their composition is quite adequate. Such limited figures do not, however, give the entire picture. The Ca and P content of herbage varies markedly during the growing season, Ca tending to accumulate and P to decline. Fertilizer application can increase both the Ca and P content of herbage, more on poor soils than on good ones, whereas drought, flooding and those differences in management that affect growth stages and botanical composition all influence the composition to a considerable extent. On occasions even a good pasture may show low values for both P and Ca, but it is very doubtful whether these short-term changes in Ca and P supply to the animal have much effect on productivity. The remaining pastures listed are all grossly deficient ones on which recognizable maladies of livestock occur. Aphosphorosis, which occurs when the P content falls below about 0·18 %, is recognized in almost every country of the world and many hundreds of thousands of square miles of grazing for cattle and sheep are affected. The loin disease of Texas, 'lamsiekte' and 'styfsiekte' of South Africa, 'bog lameness' of Ireland, the 'creeps' and 'cripples' of Australia and the 'slikkesyken' of Norway are all now known to be the result of P deficiency of the native pastures. These are nutritional diseases in the classical sense that they produce a well-recognized syndrome. There may too be even greater areas in which the highest levels of animal production are not attained owing to partial deficiency of P.

Bone abnormalities on pastures may not be solely the result of abnormalities of the Ca : P ratio or of partial deficiencies of one or other element. Rickets in sheep when grazing young cereals has been recognized in both Australia and England (Ewer & Bartrum, 1948; Ewer, 1950*a*). The disease is cured by single massive doses of Vitamin D. In Ewer's studies the vitamin D activity of the oats used was 80–120 i.u./100 g dry matter (Ewer, 1950*b*). These values are higher than the present postulated minimal vitamin D requirement of 45 i.u./100 g dry matter, and a rachitogenic principle has been postulated to be present in the herbage. Similarly, 'bent-leg' in Scotland, which Elliot & Orr (1926) showed could be cured by feeding Ca and vitamin D, was shown by Auchinachie & Fraser (1932) to be completely prevented by additional vitamin D even if the diet was very low in Ca.

Copper. It is not possible in the space available to deal with all the minor-element deficiencies that occur in native pastures, or with the suspected deficiencies due to so-called mineral imbalances, such as excessive potassium content in relation to sodium content. The Cu content of herbage has been selected to show briefly the many difficulties involved in the interpretation of analytical results from herbage.

Table 4 summarizes some analytical data for pasture herbage. It will be noted that diseases that are cured by giving Cu to the affected animal occur on pastures which differ very widely in Cu content. Only in the areas of Australia where swayback is endemic does the Cu content of the herbage fall below the estimated minimal requirement. On many pastures in Britain these Cu-deficiency diseases occur; animals show low levels of Cu in blood and liver; they respond dramatically to Cu therapy, and yet no differences in Cu content can be found between the herbage they graze and the herbage of fields on which the disease is unknown. Many reasons have been suggested for this paradox, interference with the normal Cu metabolism of the ruminant by traces of lead, zinc or molybdenum having attracted most attention. Despite considerable work, however, no explanation has yet been found.

Table 4. *Copper content of pastures in relation to the requirement of sheep and cattle*

	Cu (p.p.m. of herbage)
Estimated minimal requirement	5
Normal grazings, Great Britain[*]	7–24
Normal grazings, Australia[†]	7–12
Areas where diseases cured by administration of Cu occur:	
Swayback areas, Australia[‡]	1–4
Swayback areas, Derbyshire[§]	12–27
Swayback areas, Scotland[‖]	4·4–9
Peat scours area, New Zealand[¶]	3·6 (mean)
Peat scours area, England[**]	8–26
Copper pine areas, Caithness[††]	5–21
Falling disease areas, Australia[‡‡]	Less than 2
Polder disease areas, Holland[§§]	5–11

[*] Eden (1944).
[†] Bennetts & Beck (1942).
[‡] Beck (1941*b*).
[§] Innes & Shearer (1940).
[‖] Stewart, Farmer & Mitchell (1946).
[¶] Cunningham (1945–6).
[**] Allcroft (1946).
[††] Jamieson & Allcroft (1950).
[‡‡] Beck (1941*a*).
[§§] Brouwer, Frens, Reitsma & Kalisvaart (1938).

Fat-soluble vitamins. Vitamin D supply from pasture has already been mentioned. The grazing animal of course receives most of its protection against rickets from sunlight. In herbage crops it appears that it is largely the small dead areas of the leaf which contain vitamin D, presumably as a result of irradiation. From the scanty analyses available the vitamin D content of the pasture plants does, however, appear to be highly variable.

The enormous quantities of β-carotene present in grass are nearly always sufficient to meet requirements. Early young grass contains 300–400 times as much carotene as the animal requires, and, though the carotene content falls rapidly with advance of season, it remains above requirement. Field deficiencies do occur, however, where animals are maintained on very poor, mature, dry, bleached ranges or where such fodder is supplemented with cereals and oilcakes with very low vitamin A activity (Hart & Guilbert, 1933). Drought markedly reduces the vitamin A activity of pasture, and under such conditions vitamin A deficiency will occur (Gunn, 1942). The weather in this country precludes such deficiencies on natural pastures.

The vitamin E content of pasture grasses is quite high, but nevertheless deficiency syndromes in young lambs and young calves have been recorded. These respond to vitamin E therapy (Willman, Asdell, Grams & Hagan, 1931; Willman, Loosli, Asdell, Morrison & Olafson, 1945, 1946; Stamp & Blaxter, 1951; Vawter & Records, 1947). Analyses of food in such instances have not yet shown major deficiencies of tocopherols in the diet.

Water-soluble vitamins. Table 5 makes a comparison of requirements of animals for components of the vitamin B complex with the composition of pasture herbage in respect of these nutrients. In general, grass can be regarded as an excellent source of riboflavin, nicotinic acid, pantothenic acid, pyridoxin and biotin (the last two are not shown in the table). Grass does, however, tend to be low in aneurin. As far as ruminant animals are concerned this may be of some importance, since the evidence for the synthesis of adequate amounts of this vitamin is by no means unimpeachable (Kon & Porter, 1947–8). There is evidence too (Wegner, Booth, Elvehjem & Hart, 1940) that the level of vitamin B_1 has a decided influence on the rate of synthesis in the rumen of other members of the B complex. In this respect a disease of young cattle characterized by ataxia and abnormalities of heart action has been shown to respond to vitamin B_1 therapy in Norway (Dynna & Thune, 1941). How far the 'stravelsjuka' of cattle in Sweden is partly the result of a secondary vitamin B-complex deficiency is not known (Hoflund & Hedström, 1949). Vitamin C has not been included in this study, since it is probably not required in the diet of domesticated animals. The content in fresh young grass is extremely high (Booth, 1942).

Table 5. *Content of vitamins of the B complex in grasses in relation to the generalized minimal requirements of herbivora for these vitamins*

(All values expressed in mg/100 g dry material)

	Aneurin	Riboflavin	Nicotinic acid	Pantothenic acid
Requirement	0·1	0·15	1·0	0·5
Content in:				
Young cereals*	0·2–1·3	1·5–2·6	5·3–9·3	1·4–1·8
Mixed grassland herbage†	0·2 ± 0·01	0·9 ± 0·04	3·0 ± 0·2	0·9 ± 0·04
Lucerne including dehydrated crop		0·9–2·6‡§	6·6–15·7‖	3·3–4·6¶
	0·26**	0·9–2·6**	4·6**	2·50**
		1·67		
Poor 'grasses' (crested dog's-tail, flying bent draw moss)†	0·2–0·4	1·2–2·0	4·4–4·6	0·5–1·7
Conclusion	Lowest margin of safety	Highly adequate	Highly adequate	Adequate

* Kohler (1944).
† Thomas & Walker (1949); in the first instance mean values with their standard errors.
‡ Hanke & Perkins (1942).
§ Hunt & Bethke (1940).
‖ Hale, Davis & Baldwin (1942).
¶ Bauernfeind, Norris & Heuser (1942).
** Lardinois, Elvehjem & Hart (1944).

Toxic substances. The conversion of pastures to human food is limited not only by nutrient deficiencies of the forage. Limitations arise from the presence of toxic minerals taken up by plants from the soil and from toxic organic compounds synthesized by plants growing in the pastures. Some of the toxic minerals may be regarded as surface contaminants of the herbage, arising from soil dust or industrial fumes. Lead (Allcroft & Blaxter, 1950), fluorine (Boddie, 1945, 1947) and arsenic (Sommer, Schwarzendahl & Bergmann, 1944; Grumbach, 1944) all come in this category. Selenium poisoning which causes blind staggers in cattle in Dakota and Wyoming (Moxon & Rhian, 1943) is, however, the result of uptake of selenium by the plants themselves. In these seleniferous areas no solution to the problem has been found and hundreds of thousands of acres have been virtually abandoned by beast and man. Molybdenum poisoning, which causes the 'teart', or scouring disease, of cattle in this country, is similarly due to uptake of molybdenum from the soil (Lewis & Watson, 1942). It can be cured by administration of copper (Ferguson, Lewis & Watson, 1943), and the disease is no longer a serious problem in the areas concerned. Accumulation of nitrates in herbage, more particularly oat hay and rape, results in toxic syndromes in cattle as the result of methaemoglobin formation (Bradley, Eppson & Beath, 1940).

The number of plants poisonous to livestock is legion. In this country deaths occur every year as the result of the ingestion of bracken, ragwort, water dropwort, horsetails, hemlock and other plants. In other countries single species may cause wholesale outbreaks of poisoning. This is true of the 'geeldikkop' of the Karroo which is due to photosensitization by *Tribulus* and other plants (Quin, 1948) and of similar photosensitizing diseases which occur when species of *Hypericum* are ingested (Dodd, 1920; Seddon & Belschner, 1927–8). In Australia the presence of oestrogens in subterranean clover is sufficient to cause abortion in ewes and sexual abnormalities in castrated male sheep (Bennetts, 1947). In this country similar oestrogens have been found in pasture herbage (Bartlett, Folley, Rowland, Curnow & Simpson, 1948) and one case of comparable defects in sheep has been observed (Stamp, 1951).

Conclusions

The full conversion of the herbage of many grazings to food for man is limited. Limitations in the utilization of the proteins, fats and carbohydrates of the herbage arise primarily from mineral deficiencies of soil and pasture; to a very minor extent from vitamin deficiencies of the herbage and to an unknown extent from the presence of toxic elements and organic compounds in the herbage. Deficient pastures may support livestock at low levels of productivity; the removal or neutralization of those limiting factors at present known could, however, result in great increases in the total production of human food.

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The Direct Use of Plant Materials by Man

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It is indeed fitting that to-day's meeting should be held at the Rowett Research Institute, which is so closely linked with the name of Lord Boyd-Orr. His masterly surveys of the world food problem and its intensification by soil erosion and by increases in world population, provide the necessary background to the problems we have been discussing here.

Sir John Russell, in his presidential address to the British Association (Russell, 1949), emphasized the close relationship between land, diet and world food supplies, when he pointed out that the average area of land per head available for food production throughout the world was expected by A.D. 2000 to fall to 1 acre, or only two-thirds of the area of 1½ acres per head required at British yields to give the British prewar dietary (Table 1). Sir John, in a personal communication, kindly provided the following estimates of the average areas per head needed for different diets: three-quarters of an acre for a vegetarian diet including some milk, as in northern India, half an acre for a vegetarian diet including almost no milk, as in parts of China.

Table 1. *Areas of food-producing land required for different diets**
(*after Russell*, 1949)

Diet	Acres/head
Prewar British	1½
Vegetarian with milk	¾
Vegetarian with no milk	½
Average area expected to be available for food production in A.D. 2000	1

* Calculation based on British yields.

The low yields per acre for beef and mutton account for the high cost of our imported meat, an economic factor that is already modifying the British diet considerably. The poor conversion factors are much improved when we turn from meat to eggs and milk. Direct utilization of plant food by man does not include dairy produce but, in practice, it is very difficult to exclude all animal food from the diet. There are in this country at present over 100,000 registered vegetarians, and probably 98 or 99 % of these consume some dairy produce, providing about two-thirds of the usual intake of animal protein amongst non-vegetarians.

Nutrients supplied by a vegetarian diet

Vitamin C. Widdowson (1947) has suggested that the diet of vegetarian children tends to be deficient in vitamin C, protein and calories. Vitamin C may have been a misprint for vitamin D, since her results on the nutritive value of a week's prewar diet of seven vegetarian children, summarized in Table 2, showed the vitamin C value to be 108 %, but the vitamin D value to be 75 %, of that of normal children's diet. Adding to these results her data on another thirteen children living in a residential nursery school in Kent where they received no meat, gives an average vitamin C intake for the twenty children significantly above the normal. Findings in a week's prewar experiment on 118 vegetarian children and adults and a week's postwar experiment on about fifty vegetarian children and adults (Wokes, 1952) showed them to be receiving sufficient vitamin C.

Table 2. *Nutritive value of prewar diet of seven vegetarian children (Widdowson, 1947)*

Constituent	Nutritive value as percentage of normal *	Constituent	Nutritive value as percentage of normal*
Vitamin A	105 ± 8·3	Calcium	124 ± 17·4
Carotene	155 ± 31·6	Iron	90 ± 8·8
Vitamin B_1	104 ± 12·4	Total protein	74 ± 9·0
Vitamin C	108 ± 15·3	Animal protein	69 ± 10·0
Vitamin D	75 ± 5·9	Calories	83 ± 7·2

* Value with its standard deviation.

Vitamin D. The prewar consumption of dairy produce by vegetarians probably supplied much less than the full requirement of vitamin D (Wokes, 1941). This deficiency is not completely eliminated by the synthetic vitamin D now being added to certain foods (e.g. nut butter, cereal products). It would be interesting to discover how much vitamin D is obtained by vegetarian babies and young children through the action of sunlight on provitamin D.

Vitamin A. Vitamin A supplies also are complicated by the possible use of precursors. For children old enough to take greens or other vegetables containing provitamin A, no difficulty arises. Babies, if artificially fed, usually depend on fish-liver oils for making good deficiencies of vitamin A in their diet. Exclusion of all animal food necessitates recourse to provitamin A, of which a perfectly satisfactory source for babies does not yet seem to be available in this country, although it could no doubt be found. Red palm oil, for example, is widely used for this purpose in the tropics. Synthetic vitamin A also may soon be available in sufficient quantities.

Vitamins of the B complex. Kodicek's (1942) suggestion that a vegetarian diet might tend to be deficient in nicotinic acid has not yet proved to be correct, at least as far as this country is concerned, where vegetarians obtain from the use of wholemeal or high-extraction flours, as well as from other sources, sufficient extra nicotinic acid to make good the amount they lose by not eating meat. However, nicotinic-acid deficiencies may be encountered in other countries with diets based on cereals or pulses deficient in this vitamin. Oats and certain varieties of maize and soya usually contain much less than the minimum nicotinic-acid content of 4 μg/Cal. recently recommended by the Committee on Nutrition set up by the British Medical Association (British Medical Association, 1950). Even greater deficiencies are found in the riboflavin content of barley, maize, oats, rice, wheat and certain varieties of soya, which may be less than half the B.M.A. Committee's minimum of 0·6 μg/Cal. During World War II riboflavin-deficiency symptoms occurred in Japanese P.O.W. camps with diets with a riboflavin content of about 0·4 μg/Cal. and were in some cases ameliorated by using sprouted beans, according to an official report by Willimott (Wokes, 1952). Sprouting or germination of seeds, staple cereals and pulses increases the contents of nicotinic acid and riboflavin to different degrees depending on the germination energy involved (Klatzkin, Norris & Wokes, 1949), a fact which throws light on the long-established Eastern custom of sprouting such seeds before eating them. Vitamin B_{12} will be considered later.

Iron. Widdowson's study of the prewar diet of seven vegetarian children showed it to contain only 90 % of the normal amount of iron. This deficiency has, however, been largely eliminated by the use of flour containing a higher proportion of germ, which also helps with the supplies of various trace elements.

Energy. Results obtained by Foote & Eppright (1940) and Widdowson (1947) showed on the whole subnormal calorie intakes in vegetarian children above the age of 5. Below this age they did not differ significantly from the normal. This change at the age of 5 may be explained by the findings of Wakeham & Hansen (1932) that the average basal metabolic rate of vegetarians begins to fall when they have been vegetarians for 5 years, and eventually comes to a level about 10 % below normal.

The heights and weights of vegetarian children were found to be normal by Wakeham & Hansen (1932). Sibly (quoted by Wokes, 1952) also claimed average normal increases in height and weight in boys in one of the houses at a public school in which the diet has been vegetarian since 1909, the animal-protein content (all from dairy produce) providing about 3 % of the total calories. Data on the athletic performances (e.g. swimming, cross-country running) of the vegetarian boys, which apparently show them to be equal to the non-vegetarian boys in the same school, are being submitted to statistical analysis. It is hoped also to examine statistically the athletic records of members of the Vegetarian Cycling and Athletic Club.

Animal protein. A recent survey (Wokes, 1952) of the animal-protein intakes in different countries since 1939 shows that these have fallen considerably in many European and Asiatic countries. Whereas in North America, Uruguay and the Argentine, Australia and New Zealand the average intakes of animal protein now provide 5–8 % of the total dietary calories, in most west European countries this percentage lies between 4 and 5. In eastern Europe it is usually between 1·5 and 3, and in India and the Far East it falls below 1, and may be less than 0·5.

Economic and political factors may lead during the next few years to some smoothing out of the dietary differences between the East and the West which, in view of the diminishing area of land available per head for food production, will almost certainly necessitate further reductions in the European intakes of animal protein. Hence the experience of eastern nations who have been living for generations on very low intakes of animal food may provide useful information to the western nations facing possible reductions in their intakes of animal food. Some information of value may also be obtained from the experience of persons in this country who have been experimenting for a number of years with diets containing little or no animal food.

Protein requirements of adults

There is a good deal of evidence that adults can maintain health and activity for years on intakes of animal protein providing not more than 3 % of the total dietary calories. Similar results were obtained by McCance & Widdowson (1946) in shorter experiments, lasting 14–15 weeks, in which the corresponding percentages were 1·6–2·8 for three men, and 3·9–5·1 for two women, some of the animal protein being obtained from dairy produce and some from meat. As regards protein intake and muscular efficiency, Wishart (1934) showed in experiments on a racing cyclist who had been a vegetarian for 20 years that almost as good results were obtained with diets containing no animal protein as with diets in which as much as 18 % of the calories came from animal protein, all from dairy produce (Table 3). However, each of these diets was consumed only for 3 days.

Table 3. *Effect of animal-protein intake on external work by a vegetarian racing cyclist (from Wishart's* (1934) *data)*

Protein intake (g/day)		Percentage of calories from animal protein		Total external work (kgm × 10^3) in	
Animal	Total			6 h 24 min	8 hr 10 min
0	39	0		272	344
0	112	0		289	357
0	208	0		270	—
			Mean	277	351
191	228	16		297	381
184	213	18		289	377
			Mean	293	379

A number of adult vegetarians, in this and other European countries, have been trying during recent years to live on a diet containing no animal food. Some of them have succeeded in adhering to this drastic regime for a considerable number of years, apparently with no ill effects. The health and activity of others appeared satisfactory during the first few years of abstinence from animal protein, but after this weakness of the limbs frequently began to develop, with severe spinal pains. These deficiency symptoms do not seem to have been encountered by Germans living on diets quite low in animal protein (Members of the Department of Experimental Medicine, Cambridge, and Associated Workers, 1951). Until more is known about their cause, there would seem to be definite risks in attempting to live for prolonged periods on diets containing no animal protein, especially whilst food shortages place difficulties in the way of obtaining a sufficient variety of unprocessed foods, such as fruit and nuts. Interesting results have recently been obtained by American workers using a diet of rice and fruit providing no animal protein and only about 25 g vegetable protein daily. The body-weight, after dropping rapidly during the first few months, became steady at a lower level, when the average nitrogen balance was no longer negative. The diets have been recommended for the treatment of hypertension, but need to be maintained for much longer periods before their nutritive value can be established (Peschel & Peschel, 1950).

Protein requirements of children

The crucial test of any diet is whether it will give satisfactory results in the production and rearing of children, as well as permitting suitable growth and development and adult activities throughout the normal span of life. Records are available of families in which the members for several generations have eaten no animal food except dairy produce, their intakes of animal protein usually providing between 2 and 4 % of the total dietary calories (Wokes, 1952). Since these intakes permitted satisfactory growth of children, protein supplies must have been adequate, even although this has not been duly confirmed by nitrogen balances.

Still lower intakes of animal protein occurred in Widdowson & Dean's (1948) nutritional study, extending over a year, on German children who were given unlimited amounts of bread (with vitamin supplements) which provided 75 % of the total calories, the remainder being derived mainly from vegetables. Each child received 250–500 ml. milk per week, so that the animal-protein intake provided less than 1 % of the total calories. This study demonstrated the possibility of securing excellent growth and health on very little milk and little animal protein (McCance, 1948).

Comparison of human and cow's milk

During the early stages of lactation there is a sharp fall in the protein content of both human and cow's milk, which can be seen more clearly if it is plotted against the logarithm of the age of the baby or calf. Allowing for the shorter span of life in the cow, the rate of fall is similar in both species. The final level reached is 16–20 % in the cow as compared with about 8 % in the woman, both expressed on the calorie basis. This difference of protein levels, which is probably related to the more rapid rate of growth of the calf, means that bottle-fed babies living on National Milk are receiving much more protein than they would obtain from their mothers' milk. In many proprietary baby foods the protein content of cow's milk is reduced in different degrees by admixture with malted cereals. If cow's-milk proteins are equal in biological value to human-milk proteins, they should promote satisfactory growth in babies when supplied at the same level as in human milk, i.e. providing about 8 % of the total calories. This was shown to be possible in experiments carried out a few years ago under the supervision of Prof. Andersen of Copenhagen, at the Dronning Louise Hospital and Ordruphøj Nursery (private communication). Satisfactory growth was obtained in babies 0–8 months old deriving nearly the whole of their calories from a specially modified cow's milk in which the proteins provided only about 8 % of the total calories. Moreover, there was no significant difference in the rate of growth when the protein content was raised to about 13 % (on a calorie basis).

Vegetable substitutes for cow's milk

The shortage of supplies of cow's milk in many parts of the world has led to many attempts to devise satisfactory substitutes. These have usually been based on soya, although almonds and other nuts of high protein content have sometimes been used for this purpose. Soya beans have for many generations supplied the basis of suitable foods for babies and young children, as well as adults, in the Far East, particularly northern China and Manchuria. However, experiments on soya in India and in Europe have on the whole given less satisfactory results, the addition of some milk usually being found necessary (Aykroyd & Krishnan, 1936–7; Dean, 1949). This failure to obtain completely satisfactory growth with milk substitutes entirely free from animal protein may be due to (*a*) unsatisfactory amino-acid mixtures, (*b*) denaturation of the proteins during processing, (*c*) presence in soya of the trypsin inhibitor, which may interfere with the digestion and assimilation of the protein, or (*d*) deficiency of the vitamin B complex.

Differences in amino-acid composition between soya and human milk can be smoothed out by admixture of the soya with suitable proportions of cereals (Wokes & Klatzkin, 1949). Denaturation changes can be reduced to a minimum by processing in vacuo. The trypsin inhibitor can be eliminated by means of germination enzymes, using malted cereals and soya as the raw materials. But the malting must be carried out in such a manner as to secure adequate development of the enzymes as well as of the vitamin B complex. If such precautions are not taken these milk substitutes may be definitely deficient in riboflavin. This deficiency can be particularly serious in very young babies because of the need for high levels of riboflavin in the earliest weeks of life.

The animal protein factor

Recent experiments on various animal species have shown that plant proteins give better growth and development if supplemented with the animal-protein factor, which appears to be closely related to vitamin B_{12}. As far as human diets are concerned, evidence of the need for this factor is still unconvincing, experiments on American children having given varying results (Wetzel, Fargo, Smith & Helikson, 1949; Downing, 1950). This may be due to the presence of stores of the vitamin in the body tissues when the experiments began. Such a difficulty might be overcome by choosing as experimental subjects the babies of mothers who have abstained from animal food not only whilst breast feeding the babies but also for some months before the babies were born. The breast milk of such mothers should have a very low content of the animal protein factor. Their babies are often found to have allergies to dairy protein which may be associated with asthma and cause much difficulty in experimentation.

In examining the results of these experiments on milk substitutes it should be borne in mind that the rate of growth in children on diets very low in animal protein is often below normal, according to records kept over a number of years by my colleague Dr Cyril Pink. Nevertheless, such children are usually able to maintain satisfactory activity and development without increase in their animal-protein intake. In such instances the rate of growth may need correlating with other criteria when the nutritive value of the diet is assessed.

In conclusion, the opinions of Lord Boyd-Orr and Sir John Russell (1949) indicate that the problem we have been considering here is ultimately concerned with the future of the human race. In the short space at my disposal only brief reference could be made, in fairly general terms, to a few of its more important aspects. The results so far obtained are affected by so many factors that they will need repeating over and over again before conclusive findings, that are statistically significant, can be reached. Nevertheless, in spite of many failures and disappointments, the work is important enough to ensure that the necessary refinement of experimentation will sooner or later be secured. The generous help and encouragement of my friends and colleagues lead to the hope that our faith in the future of mankind will eventually be justified.

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The Nutrition of the Young Ayrshire Calf

8. Muscular Dystrophy in the Growing Calf

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(*Received* 27 *March* 1951)

In earlier work on the nitrogen metabolism of the young calf (Blaxter & Wood, 1951 *a*, *c*) the sole source of vitamin E in the diets was high-quality dried skim milk that had been packed in sealed tins. At the time it was thought that this source of vitamin E activity would be sufficient to protect the animal during the short-term experiments envisaged, especially in view of the very considerable difficulties that have been met in devising cattle diets containing little vitamin E (Gullickson, Palmer, Boyd, Nelson, Olson, Calverley & Boyer, 1949; Gullickson & Calverley, 1946). One of our early experiments, however, had to be abandoned owing to the onset of a syndrome in which vitamin E appeared to be involved.

Six animals were affected 24–60 days after birth. The animals became very weak and unwilling to stand for more than a few minutes; the pastern joints became straightened; the hind-legs crossed when attempts to walk were made; there was a general ataxia, and the neck was outstretched in a very characteristic way. A tetany, not involving the extremities, was common during the latter stages and was seen only when the severely affected animal was placed on its feet. Relaxation of the musculature of the throat and inability to control movements of the tongue made drinking difficult and, in the terminal stages, the calves choked, owing to liquid food entering the trachea. Loss of hair at the bulbal base of the ear was always a common feature; the coat was staring and the skin thickened, the latter making superficial venesection difficult. In two animals oedema of the brisket and ascites were present. This was of rapid onset as judged by gains in body-weight and seemed to be associated with a slight exophthalmos. In these oedematous calves the pulse was highly erratic and generally increased in frequency. Death occurred suddenly if such signs of cardiac abnormality were present; otherwise the animals died following a period of prostration. At post-mortem examination ascitic fluid was found in the abdominal cavity, the carcass was generally slightly oedematous, and one constant feature was the rapidity of putrefactive change. The liver and kidneys were normal; the lungs were pneumonic and contained milk and blood. The heart was dilated in some animals, and slight white lesions were found in the walls of the left ventricle in one of those animals affected by oedema. The bones were calcified quite normally but some erosion of the articular cartilages was present. This, however, was also found in normal calves. The skeletal muscle was very pale and flabby, and the fat was a light brown compared with the normal whitish yellow colour. Before death, and when clinical signs were present, blood samples were taken and normal serum values for calcium, inorganic phosphorus and magnesium were found. There was no response to magnesium sulphate given intravenously, suggesting that the tetany and prostration were not the result of hypomagnesaemia, i.e. 'milk' tetany of the calf as described by Duncan, Huffman & Robinson (1935). Urinary excretion of vitamin B_1 was not affected, thus excluding aneurin deficiency. The only biochemical abnormalities found were an elevation of the urinary elimination of creatine from normal values of 200–300 mg/24 h to as much as 1·3 g/24 h, and an elevation of basal oxygen consumption by 20–60 %. This syndrome and the post-mortem findings appeared comparable to those obtained by Goettsch & Pappenheimer (1931) in guinea-pigs given diets deficient in vitamin E. A diagnosis of avitaminosis E was made, and attempts to cure two affected animals followed. In one calf, no response occurred following the daily administration of 25 mg DL-α-tocopherol. An aqueous solution of 100 mg α-tocopheryl phosphate was then given for 5 days. Improvement was rapid; from complete prostration the calf's behaviour changed markedly so that it could jump and frisk in its cage; the balance improved but it still found difficulty in rising. This was undoubtedly due to bruising of the hocks, stifles and knees during the 14 days' prostration. The creatine content of its urine fell from nearly 600 to 270 mg/day, and on slaughter its musculature appeared normal. A further calf, however, when severely affected was given 100 mg α-tocopheryl phosphate daily for 10 days without response. In this animal, there was an acute alimentary disturbance and a pneumonia which did not respond to sulphonamide therapy. The creatine content of a sample of skeletal muscle was very low and the extent of the muscular involvement was so great that a cure could hardly have been expected. Thus in only one of the two calves was a cure effected.

Later, histological examination of the tissues from two of the calves showed a degeneration and inflammatory infiltration of the cardiac and skeletal muscles, and in the spinal cord a degenerative process of the neurones associated with oedema and gliosis (proliferation of the neuroglia). Affected skeletal muscle showed granular degeneration and fragmentation of muscle cells and their replacement by fibroblasts, endothelial cells and plasma foci. Nuclear proliferations were also present. Cardiac muscle from the left ventricle also showed grossly degenerate and granular myocardial and endothelial cells and replacement by fibroblastic tissue. The Purkinje fibres were swollen.

These findings were not completely comparable to those observed in rabbits where muscular dystrophy is associated with replacement of normal muscle cells by fatty tissue (Pappenheimer, 1948) and where nervous tissue does not appear to be affected, but the nature of the whole syndrome suggested a deficiency of vitamin E.

Muscular and other disorders associated with dietary deficiencies of vitamin E have been reviewed by Pappenheimer (1940–1, 1943, 1948), Follis (1948), Parrish (1949) and Loosli (1949). Muscle lesions have been shown in rabbits and guinea-pigs (Goettsch & Pappenheimer, 1931), mice (Pappenheimer, 1942), rats (Knowlton & Hines, 1938; Pappenheimer, 1939), ducks (Victor, 1934; Pappenheimer & Goettsch, 1931), monkeys (Mason & Telford, 1947) and dogs (Anderson, Elvehjem & Gonce, 1939). Even with chicks in which vitamin E deficiency manifests itself primarily as an encephalomalacia, abnormalities of the musculature are nevertheless present (Pappenheimer, 1948).

In cattle, the recent studies of Gullickson and his collaborators (Gullickson & Calverley, 1946; Gullickson, 1949; Gullickson *et al.* 1949) have shown that vitamin E deficiency is associated with sudden death of cattle resulting from cardiac failure. Electrocardiography showed a decreased functional activity of the myocardium. At post-mortem examination no gross pathological changes were found, and a short report on the histology of the heart indicated that the condition resembled human endocarditis. No mention was made by Gullickson of any dystrophic lesions of either heart or skeletal musculature in these animals, although they had subsisted for as long as 5 years on diets very low in vitamin E. In 1947, however, Vawter & Records (1947) discovered a series of farms in Nevada, U.S.A., on which calves on the range died with symptoms of dystrophy closely comparable to those associated with 'stiff lamb disease' (Willman, Asdell, Grams & Hagan, 1931), a disease known to be cured by DL-α-tocopheryl acetate (Willman, Loosli, Asdell, Morrison & Olafson, 1945, 1946). Vawter & Records (1947) were unable to show that this condition was a true muscular myopathy caused by vitamin E deficiency, but they drew attention to the close resemblance of the disease to muscular dystrophy induced in other species by dietary deficiencies of vitamin E.

There are numerous records in the literature of muscle and heart lesions in young calves that appear comparable to those described by Vawter & Records. Most, but not all, of these reports refer to the toxicity of cod-liver oil. One of the earliest descriptions of the disease was by Slagsvold (1925), though Agduhr (1927) claims to have observed it first in 1922. Later, Hjärre & Lilleengen (1936*a*, *b*) traced records of a disease called *weisses Fleisch* to the latter part of the nineteenth century, and one of the earliest reports was that of Pollock (1854). The symptoms are well summarized by Hjärre & Lilleengen. The same authors have attributed the naturally occurring muscular dystrophies of calves to vitamin C deficiency on the basis of the appearance of the disease when heated oxygenated milk was given. Such methods destroy not only vitamin C but vitamin E as well, and in view of the absence of dietary requirement of vitamin C in the ruminant such an explanation does not seem likely.

The many reports of Agduhr incriminating cod-liver oil (Agduhr & Strenström, 1929) stimulated considerable work on other animals. Dystrophy was produced in sheep and goats given cod-liver oil in large amounts (Madsen, McCay & Maynard, 1935) and also in pigs (Wagener, 1936), though attempts to confirm it in pigs were not successful (Scorgie & Miller, 1944). With calves the experiments to confirm the European reports were in all cases unsuccessful. Davis & Maynard (1936, 1938) reared calves on rations containing cod-liver oil, but the only animal that died showed no evidence of dystrophy. Later experiments showed that the electrocardiograms of such calves were normal (Barns, Davis & McCay, 1938). Experiments with seventy-two calves by Gullickson & Fitch (1944) in which 25–35 ml. cod-liver oil were given every day did not reveal any adverse effects. Turner, Meigs & Converse (1936) briefly reported death of a calf or calves when 2·0 g cod-liver oil/kg body-weight (possibly as much as 100 ml./day) were given, but the general contention has been that muscular dystrophy cannot be produced by giving cod-liver oil to calves. Experiments in other species, however, have confirmed that cod-liver oil can destroy vitamin E (Cummings & Mattill, 1930–1), and with cows Ferrando, Chenavier & Cormier (1949) have shown that administration of cod-liver oil depresses the content of tocopherols in the blood plasma.

In view of these reports and our own preliminary investigations, an experiment was planned to provide further information. The object of the experiment was two-fold. Firstly, to find whether the inclusion of vitamin E in a diet containing minimal quantities of vitamin E would prevent muscular dystrophy; and secondly, to find whether the addition of cod-liver oil to this diet would result in the incidence of a more severe form of the disease.

EXPERIMENTAL

Plan of experiment

Calves. Sixteen Ayrshire bull calves, purchased from the market when 2–3 days old, were used as experimental animals. Eight were purchased in January 1950, and eight in July 1950. They were confined in individual pens and were bedded on straw or peat moss in a room thermostatically controlled between 60 and 65° F. After a short preliminary period to allow the calves to become accustomed to their new home, they were grouped into pairs on the basis of their age and general well-being and the pairs were then allocated at random to one of two diets. The diets were similar in every respect, save that in one vitamins A and D were supplied as cod-liver oil and in the other as the pure vitamins in equivalent quantity dissolved in arachis oil. One animal of each pair was then allocated at random to receive a supplement of α-tocopherol, the other receiving none. There were thus four treatments, the cod-liver oil diet without α-tocopherol (CLO.O.) the cod-liver oil diet with α-tocopherol (CLO.E.), the arachis-oil diet without α-tocopherol (AO.O.) and the arachis-oil diet with α-tocopherol (AO.E.). The calves are referred to by treatment and replication number. Thus calves nos. CLO.E. 1 and CLO.O. 1 were the first pair of animals receiving the cod-liver oil diets, whereas calf no. AO.E. 4 and calf no. AO.O. 4 were the last pair of animals receiving the arachis-oil diets. Pairs of animals were always treated in exactly the same manner, save for the fact that one received α-tocopherol. In each series of eight, two pairs of animals received the cod-liver oil diet and two pairs the arachis-oil diet.

Diets. The basic diets were almost the same in each experiment, the only difference being that the second eight calves received additional glucose. The composition of the diets is given in Table 1. The first group of eight calves were given 3·6 l./day for the first 14 days and 4·6 l. subsequently. The second group of eight were given 4·0 l. until they were 60 days old when the quantity was increased to 4·8 l. In the second group, however, the concentration of arachis oil or cod-liver oil was reduced when the ration was increased so that the calves ingested the same quantities of vitamins A and D as when 4·0 l. of diet were given. With these diets the amount of arachis oil or cod-liver oil taken daily was 15–18 ml. This quantity is considerably less than that recommended for calf rearing by the Ministry of Agriculture and Fisheries (1937). The dried skim milk was of high quality and high solubility. It had been packed in the factory in sealed cans, and these were opened as required. The lard was household-quality lard. The cod-liver oil was purchased from recognized veterinary suppliers and was veterinary quality refined oil with a guaranteed potency. Both the arachis-oil solution of vitamins A and D and the cod-liver oil were stored in a refrigerator. The α-tocopherol supplement given to the calves in groups CLO.E. and AO.E. was the synthetic racemic α-tocopheryl acetate. It was also refrigerated, and 50 mg dissolved in 0·2 ml. arachis oil were given daily in a small gelatin capsule.

Table 1. *The basic diet of the calves*

Constituent	Quantity/l.
Spray-dried skim-milk powder	82·5 g
Lard	41·2 g
Glucose (second series only)	13·7 g
'Trace'-mineral solution*	2·5 ml.
Cod-liver oil, or arachis-oil solution of vitamins A and D	4·1 ml.

* Containing 1·25 mg $MnSO_4.4H_2O$, 1·25 mg $CuSO_4.5H_2O$, 0·25 mg $CoCl_2$, 50 mg ferric citrate and 150 mg $MgCl_2$.

The diets were made by methods previously described (Blaxter & Wood, 1951*a*) twice weekly at intervals of 3 and 4 days and were stored in an immersion cooler at 5–7°.

Management of the calves. The calves were fed at 7 a.m. and 5 p.m. The α-tocopherol supplement was always given at 2 p.m., between normal feeding times. When the calves became unable to drink they were suckled.

After some time in their pens the calves, especially those that had grown well, began to eat their straw bedding. This was immediately controlled by muzzling them with leather greyhound-muzzles except when they were fed. After a further period, however, the calves learned to eat straw through these muzzles. Peat moss was then substituted for the straw but, even so, the calves managed to consume some of it, and peat moss was commonly found in the rumens of the older calves when they were slaughtered, but the amounts involved were small.

Examination of the calves during life. Notes on the animals' behaviour were made twice daily, and when they were weighed at weekly intervals the calves were exercised on a cement floor and abnormalities of gait noted. The time taken to consume their food was recorded at intervals, and urine specimens (not 24 h samples) were also collected. Animals nos. AO.E. 3, AO.O. 3, AO.E. 4 and AO.O. 4 were subjected to balance experiments for a period of 14 days once signs had become apparent, using the technique of Blaxter & Wood (1951*a*). Oxygen consumption and carbon-dioxide production were determined after an 18 h fast, using the technique of Blaxter & Howells (1951).

Post-mortem examination of the calves. When the symptoms of muscular dystrophy were judged to be sufficiently advanced in any calf it was killed for dissection, pathological examination, and chemical analysis. Its corresponding pair-mate was killed 3–6 days later and the same routine repeated.

Cultures were taken from any organs that appeared abnormal. One fore-leg and one hind-leg from each animal were dissected in detail, and a description was recorded of any lesions present in each of fourteen muscles, representing the main muscles of the limbs. These muscles, dissected free of fat, tendon and muscle sheath, were subsequently used for chemical study. Notes on the abnormalities of other skeletal muscles were also made, and the weights of the liver, brain, kidneys and heart were obtained.

RESULTS

Signs of muscular dystrophy shown by the calves

Group AO.E. Arachis-oil diet with α-tocopherol

All calves in this group behaved normally throughout the experiment. On several occasions no. AO.E. 2 and more especially no. AO.E. 1 were affected by an acute diarrhoea, whereas no. AO.E. 4 was but slightly affected at any time. No abnormalities of gait or balance were noted in the calves, and their coats were sleek, except when affected by diarrhoea. Calves nos. AO.E. 3 and AO.E. 4 had gained over 20 kg by the time they were killed, corresponding to a daily gain of 230 g. Calves nos. AO.E. 1 and AO.E. 2, however, did not gain as much, owing to the incidence of alimentary disturbance. A photograph of a normal calf (no. AO.E. 3) is given in Pl. 1, 1.

Group AO.O. Arachis-oil diet without α-tocopherol

Calf no. AO.O. 1. At the 10th day of treatment this calf was thought to be quite normal. On the 16th day, however, the eyes were sunken, the coat was rough, and on standing the legs flexed at the knees. On walking, the hind-feet were placed in a crosswise fashion and the hock joints were consequently twisted outwards. Breathing was laboured and muscles of the tongue and throat soon became useless. Pneumonia was diagnosed and the calf was killed. It was obvious in this calf that a partial paralysis of throat muscles and interference with swallowing reflexes contributed to its condition. Over the whole period, including the terminal phase, no gain in weight had occurred.

Calf no. AO.O. 2. No abnormalities were observed until the 25th day of treatment when muscular co-ordination seemed abnormal; skin reflexes, however, were unaffected. The condition deteriorated until the 30th day when violent tremors and shivering were observed. The calf stood with an arched back and had to be helped to its feet. The hind-legs appeared weak. During this period the faeces were quite firm. After the 34th day an improvement occurred, but on the 38th day further abnormalities of the gait were seen, consisting of forward flexion of the knees in a manner similar to that observed in calf no. AO.O. 1. Signs then became more pronounced. The coat became rough, the calf dull and lethargic, a slight cough developed on the 44th day. By the 47th day the calf stood with all four feet bunched together, with the back elevated and the head held very low. It could only rise with difficulty and could only drink with considerable effort. The calf was killed on the

51st day having lost weight severely during the last week of life. The overall weight gain was only 100 g/day.

Calf no. AO.O. 3. Nothing abnormal was noted until the 23rd day when the calf was listless and had a discharge from the nose, diagnosed as a slight cold. By the 31st day the cold had gone, but the calf spent very much longer lying down than he had done previously or than his pair-mate no. AO.E. 4 did at the time. This lethargy continued until the 50th–52nd day when the gait appeared slightly stiff. Before this, both gait and balance had been perfectly normal. A week later the shoulders appeared to be abnormal, and on running the animal often stumbled and fell as the result of failure to flex the fetlock joint. This state continued until the 76th day, when coughing after meals was observed and the constant lying down and listlessness appeared again. The claws of the hoof were spread considerably, and by the 83rd day there was difficulty in rising. The calf was slaughtered on the 91st day without further exacerbation of these signs being noted. It had gained 200 g/day throughout.

Calf no. AO.O. 4. Nothing abnormal was noted until the 39th day when the time taken to consume a meal was prolonged. On the 54th day the calf was very quiet, and on the 55th day the shoulders were noted to be abnormal. This abnormality persisted until death, increasing markedly with increase in weight. Slowness in drinking and constant lying down were the only other symptoms observed. Slight coughing after feeding was noted on only one occasion. The calf was killed on the 98th day and had gained 210 g/day.

Group CLO.E. Cod-liver oil diet with α-tocopherol

Calf no. CLO.E. 1. This calf was quite normal until the 17th day when the gait was staggering and the fetlocks flexed. These signs did not get worse, and nothing further was noted until the 36th day when the time taken to consume food was excessively prolonged. The next day the calf appeared stiff, and muscle tremors occurred on standing. It was killed on the 38th day since the other calf of the pair had already succumbed.

Calf no. CLO.E. 2. When the experiment commenced this calf had a tendency to flex the knee joint of the left leg, a sign which in the preliminary experiments had been associated with dystrophy. This grew worse throughout the experiment, but in a periodic fashion. Thus, by the 21st day the calf, on rising, would fall forward and the knees would flex, but by the 31st day it was able to rise unaided and the front legs became bent only after prolonged standing. The signs became worse again on the 40th day when it could straighten the right leg but not the left, and its balance was markedly affected. The calf rarely moved the hind-legs when in its pen, but when exercised moved them stiffly. It could walk quite quickly on its permanently flexed front legs. It was killed on the 45th day. A photograph of this calf is given in Pl. 1, 2.

Calf no. CLO.E. 3. Except for showing bouts of nervousness, this calf remained perfectly normal for the first 44 days. On the 45th day it became rather quiet and very slow in drinking. The habit of standing with its hind-quarters in the corner of its pen was adopted, and on exercise marked ataxia was present. A slight cough developed on the 48th day and the animal lay down at every opportunity. The shoulders and back were normal but the gait was slightly unsure and the feet were placed farther forward than is normal on walking. It was killed as a pair control on the 55th day without further signs developing.

Calf no. CLO.E. 4. This calf remained normal throughout the experiment save that it was lethargic 2 days before slaughter on the 35th day, the pair-mate having previously succumbed.

Group CLO.O. Cod-liver oil diet without α-tocopherol

Calf no. CLO.O. 1. This calf was normal for the first 14 days. By the 23rd day, however, it was very slow to rise, and found difficulty in drinking. Shivering on standing was noticed on the 27th day and it was not able to suck properly. The front legs became stiff, and flexion of the knee joints was absent. On the 29th day respiration was slightly pneumonic, and when lying the calf adopted unnatural attitudes. On the 33rd day it could stand only for a few minutes and with violent muscle tremors and seemed unable to use the tongue or lips, or to be able to swallow. The calf was killed on the 34th day, and at death its weight had fallen to the initial weight, though, up to the onset of symptoms, growth had been normal.

Calf no. CLO.O. 2. This calf, apart from a slight cold and a disturbance of the alimentary tract, was normal for the first 41 days. On the 41st day a tendency to flex the carpal joint of the right fore-leg was noted. By the 46th day drooping of the head and a rough coat were the predominant signs. On the 65th day just before slaughter both fore-legs were flexed at the carpus, and stiffness of the hind-limbs was noted.

Calf no. CLO.O. 3. This calf was normal for the first 25 days, except for slowness in drinking and loss of hair from the bulbal base of the ear. Slight coughing was noted, which became worse until the 36th day. During this period there was no increase of rectal temperature; the calf was lively and his gait normal save that when pushed he was slow to regain his balance. A rough coat and prolonged drinking time were the next signs observed, and by the 43rd day an elevation of the suprascapular cartilage was evident. On the 45th day the calf was very quiet, the respiratory rate was increased but there was no rise in rectal temperature. The following day it lay down at every opportunity but could rise easily, although the suprascapular cartilage had risen still further. The next day great difficulty in rising was observed, and on the following day (the 48th) the hind-legs were tucked under the body on standing and the fore-legs were straight. Violent rigors occurred on standing but not when lying. The pulse rate and rectal temperature were normal but the respiratory rate was elevated. A million units of penicillin were given without response, and the calf was killed on the 49th day. It had gained 260 g/day during the period, a normal amount as judged by the calorific value of the diet.

Calf no. CLO.O. 4. Nothing abnormal was noted in this calf until the 16th day, when it was sluggish and inclined to lie down a great deal. By the 21st day, though able to rise and stand, it would lie down immediately after each meal. Nothing more specific was noted until the 24th day when the back was arched and the feet were flat, the latter owing to relaxation of the carpal joint. Violent shivering attacks occurred and the calf appeared breathless. The following day the calf had a great struggle to get to its feet, and on standing shivered violently and was unable to remain standing for long. A slight cough was present. The following day it could rise only if assisted, and was unable to use the tongue for drinking or sucking. It was killed on the 29th day, having gained 130 g daily over the period, this low gain being partly due to failure to consume food towards the end of life.

General observations

From these case reports, it will be seen that the same signs of muscular weakness occurred in several of the calves as occurred previously in preliminary experiments, and the same variation in time of onset of symptoms was apparent. Signs of failing circulation, however, were only observed in one calf, no. CLO.O. 3 (*vide infra*). Table 2 summarizes the severity of the signs of muscular involvement as judged by the daily observations. It will be noted that the most severe signs were observed in the calves given cod-liver oil and deprived of vitamin E.

Table 2. *Summary of treatment and signs* of the calves*

No. of calf in group	Vitamins A and D supplied by			
	Cod-liver oil		Solution in arachis oil	
	With vitamin E (CLO.E.)	Without vitamin E (CLO.O.)	With vitamin E (AO.E.)	Without vitamin E (AO.O.)
1	First signs at 36 days Killed at 38 days Slight	First signs at 27 days Killed at 34 days Severe	Normal Killed at 24 days Nil	First signs at 16 days Killed at 18 days Slight
2	First signs at 21 days, relapse at 40 days Killed at 45 days Severe†	First signs at 41 days Killed at 46 days Severe	Normal Killed at 44 days Nil	First signs at 25 days, relapse at 38 days Killed at 44 days Severe†
3	First signs at 43 days Killed at 55 days Slight	First signs at 43 days Killed at 49 days Very severe†	Normal Killed at 95 days Nil	First signs at 50 days Killed at 91 days Slight†
4	Normal Killed at 35 days Nil	First signs at 24 days Killed at 29 days Very severe†	Normal Killed at 101 days Nil	First signs at 59 days Killed at 98 days Slight†

* Signs are roughly classified as nil, slight, severe and very severe on the basis of the case reports.
† First animal of pair to be killed.

The syndrome in the affected animals always started in the same way: a disinclination to stand, together with non-specific dullness and listlessness. More specific signs appeared later. Forward bending of the knee joints occurred in some animals, and is illustrated in Pl. 1, 2 (calf no. CLO.E. 2). In these calves the feet were placed far forward on the ground, and in calf no. CLO.E. 2 the metacarpal bone made an angle of 30° with the ground. In two calves similar bending of the fetlock joint was noted. Another common symptom was the retraction of the fore-limbs under the body, resulting in the calf standing on its toes. A photograph of a calf in such a position is given in Pl. 1, 3 (calf no. CLO.O. 3). In the second part of the experiment the protrusion of the scapula was noted. Pivoting on the glenoid cavity, the scapula moved to a more upright position dorsal to the neural spines of the thoracic vertebras. This left a hollow between the two scapulas and the back of the animal. A photograph of an affected calf is given in Pl. 1, 4 (calf no. AO.O. 4). This sign is comparable to winged scapula in human muscular dystrophy of the scapulo-humeral type as illustrated by Bicknell & Prescott (1946). Relaxation of the musculature of the foot, an increase in the slope of the pastern and coronet bones and a splaying of the hoofs were also noted. In the hind-limbs the tibia and metacarpal bones assumed a straight line and were moved stiffly together. A characteristic outward swinging of the hock joints was noted in some calves, but in general the hind-limbs did not appear to be so severely affected as the fore-limbs. A photograph of a normal calf (calf no. AO.E. 3) is given in Pl. 1, 1 for comparative purposes. Each one of these peculiar postures and gaits appeared to be the result of an adjustment to minimize strain on part or parts of the skeletal musculature. This aspect is discussed later. The shivering and tetany appeared to be an 'effort' tetany rather than an irritability phenomenon. Skin reflexes were always present, but attempts to elicit deep tendon reflexes in any of the calves, normal or affected, were not very successful. An involvement of the nervous system could not therefore be discounted. Attempts were made to localize the muscular abnormalities by subjecting the animals to stress. Weakness of the suspensory muscles of the neck could be diagnosed in a comparative fashion by placing the hand on the head of the calf and pushing downwards. Similar methods were adopted for the hind-quarters and fore-quarters. Palpation of the muscles was extremely difficult. In young dairy animals the musculature is not well developed, and the only muscles that could be palpated with ease were the triceps group in the fore-limb and the vastus group in the hind. There was no indication of any enlargement or atrophy of these muscles in any calf, but the normal resilience of the muscular tissue was reduced in badly affected animals such as no. CLO.O. 3 and the muscles felt leathery. A diagnosis of dystrophy made in this way, however, was not infallible.

One common sign was the difficulty the calves experienced in drinking their food. Table 3 summarizes the results of a series of observations on the eight calves of the second series of experiments. It will be noted that affected calves took considerably longer to consume their food. Eventually it became impossible for seriously affected calves to drink at all and they had to be suckled. At no time was it noted that the sphincters of the bladder or bowel were affected.

Nitrogen metabolism of the calves

The mean daily nitrogen balances of calves nos. AO.E. 3, AO.E. 4, AO.O. 3 and AO.O. 4 are given in Table 4. They apply to 10-day experiments made when the calves were 80 days old. Those calves that did not receive vitamin E stored less nitrogen than their respective pair-mates. In Fig. 1, the nitrogen balances are plotted against the mean creatine content of the musculature as determined at post-mortem examination. The amount of nitrogen stored daily was related to the creatine content of the musculature; those calves that stored most nitrogen had most normal muscles as judged by their creatine content.

Table 3. *Time (sec) taken by each of eight calves to consume 2·0 l. of the diet*

	Vitamins A and D supplied as							
	Cod-liver oil				Solution in arachis oil			
	With vitamin E		Without vitamin E		With vitamin E		Without vitamin E	
Day	CLO.E. 3	CLO.E. 4	CLO.O. 3	CLO.O. 4	AO.E. 3	AO.E. 4	AO.O. 3	AO.O. 4
36	40	42	108	125	36	42	64	60
43	89	50	78	135	30	31	45	39
46	95	52	100	—*	30	32	65	64
57	95	—†	97	—*	32	25	35	58
60‡	90	—†	95	—*	40	42	53	96
70‡	72	—†	132	—*	26	30	30	77
Mean	97	(46)	102	(130)	32	34	49	66
Symptoms of dystrophy	Slight	Nil	Very severe	Very severe	Nil	Nil	Slight	Slight

* Killed as severely dystrophic. † Killed as pair-mate to no. CLO.O. 4. ‡ 2·4 l. given.

Table 4. *Mean daily nitrogen balances of the calves*

(The average quantity of nitrogen ingested by each calf was 22·00 g/day)

		Nitrogen excreted		
Calf no.	Treatment	Faeces (g)	Urine (g)	Nitrogen stored (g)
AO.E. 3	Given vitamin E	2·42	9·94	9·64
AO.E. 4		1·44	8·82	11·74
AO.O. 3	Deprived of vitamin E	2·87	10·73	8·40
AO.O. 4		3·16	11·60,	7·24

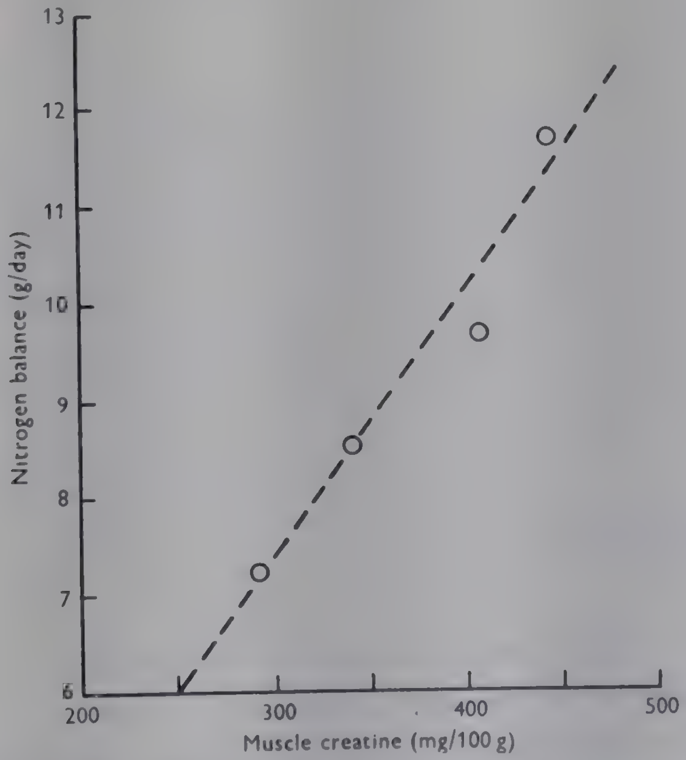

Fig. 1. Relation between the mean muscle creatine of the calves and their nitrogen balances determined immediately before slaughter. The muscle creatine refers to the mean content of fourteen appendicular skeletal muscles.

In Table 5 mean values are given for the excretion of nitrogenous metabolites by these four calves. The greater excretion of nitrogen in the urine of the calves deprived of vitamin E was traced to a greater excretion of the total of urea, ammonia and amino-acid nitrogen. The total excretion of creatinine nitrogen was lower in the vitamin E-deficient animals, suggesting a smaller functional muscular mass. Though dystrophy was present in the carcass of calf no. AO.O. 4, a creatinuria was not observed. A creatinuria in which the ratio creatine nitrogen : creatinine nitrogen was greater than 1·0 was noted, however, in calf no. AO.O. 3. Dystrophy was very advanced in the former animal, and it is possible that a creatinuria is not diagnostic in such advanced cases.

Table 5. *Mean daily excretion of nitrogen (g) in different nitrogenous metabolites by the calves*

	Calves given vitamin E		Calves deprived of vitamin E	
Nitrogenous metabolite	AO.E. 3	AO.E. 4	AO.O. 3	AO.O. 4
Protein	0·38	0·26	0·32	0·33
Urea	4·79	4·53	5·74	7·14
Ammonia	0·81	0·56	1·12	0·91
Amino-N	0·36	0·26	0·31	0·24
Total urea, ammonia and amino-N	5·96	5·53	7·17	8·29
Purine base	0·18	0·16	0·15	0·19
Uric acid	0·05	0·03	0·04	0·06
Allantoin	0·70	0·61	0·76	0·89
Total purine	0·93	0·80	0·95	1·14
Creatinine	0·63	0·60	0·34	0·43
Creatine	0·20	0·29	0·38	0·20
Total creatine	0·83	0·89	0·72	0·63

Energy metabolism of the calves

Six determinations of metabolism were made. In only two instances, however, were the calves badly affected at the time of the determinations. The heat production of the affected calves, nos. CLO.O. 3 and CLO.O. 4, was 63·5 and 59·4 Cal./kg body-weight/24 h compared with normal values of 45–49 obtained with the other calves. An increase in basal heat production would account for an increase in urinary nitrogen excretion limited to the urea, ammonia and amino-nitrogen fractions, as has been shown elsewhere (Blaxter & Wood, 1952). An increase in basal metabolism in vitamin E deficiency has been demonstrated in intact rats by Biddulph & Meyer (1942) and an increase in the metabolic rate of isolated muscle from animals deficient in vitamin E is well known (Mason, 1944).

Post-mortem examination of the calves

Group AO.E. Arachis-oil diet with α-tocopherol

Calf no. AO.E. 1. The internal organs all seemed normal and no growth was obtained from cultures of spleen and bone marrow. The heart showed some sub-endothelial valvular haemorrhage, probably comparable to the haematomas observed by Florence (1922). Their occurrence in calves can be regarded as normal. The skeletal musculature was pale.

Calf no. AO.E. 2. A small area of pneumonia was detected in the right apical lobe. There was no bronchitis, but very slight gelatinous adhesions. Cultures from the lung showed *Bacterium neapolitanum* to be present. The skeletal musculature was normal.

Calf no. AO.E. 3. This calf was completely normal in every respect, save that the thymus was unusually large. The skeletal musculature was normal.

Calf no. AO.E. 4. Apart from an apparent cavitation of the right adrenal gland all internal organs were normal. The skeletal musculature was normal.

Group AO.O. Arachis-oil diet without α-tocopherol

Calf no. AO.O. 1. On examination, the organs of this animal appeared normal. Subendothelial auriculo-ventricular haemorrhages were present, but the heart and skeletal musculature appeared normal.

Calf no. AO.O. 2. An area of pneumonia was found in the right apical lobe, spreading to the left and right cardiac lobes. Subendothelial haemorrhages were present in the heart, but the muscle appeared firm. The bone marrow was pale. The musculature was generally pale and some muscles showed white areas, more especially the supraspinatus and infraspinatus. The degree to which the muscles were affected, however, was not severe.

Calf no. AO.O. 3. A very slight pneumonia was found in the right apical lobe of the lung. This could not be regarded as serious. The heart showed slight inflammation of the pillars of the left ventricle. Two white areas were found in the rectus femoris, and further areas in the vastus lateralis. Otherwise the muscles, though pale, appeared normal.

Calf no. AO.O. 4. The carcass of this calf had a peculiar and unpleasant sweetish odour. Pneumonia was present in the right apical lobe. The internal organs were otherwise normal. The heart muscle was grossly dystrophic, showing large white areas and two areas of haemorrhage in the wall of the left ventricle. A sketch of these lesions is shown in Fig. 2. The skeletal musculature was also dystrophic. Not all muscles were affected. The neck and facial muscles appeared to be those most affected. The supraspinatus, infraspinatus, and long heads of the triceps appeared to be the most seriously affected parts of the fore-limb. In the hind-leg, the gastrocnemius seemed most affected.

Group CLO.E. Cod-liver oil diet with α-tocopherol

Calf no. CLO.E. 1. A consolidated area of congestion was present in the right apical lobe of the lung and some petechiation of the muscle ridges of the bowel wall was noted. The heart appeared flabby but otherwise normal save for auriculo-ventricular haemorrhages. The skeletal muscles were dystrophic. The biceps femoris was completely white in colour and markedly oedematous. Other muscles were not so severely affected, some appearing normal and others showing a patchy distribution of white areas. The supraspinatus was markedly haemorrhagic, whereas other muscles merely appeared pale.

Calf no. CLO.E. 2. No pneumonia was noted and the internal organs were normal. The heart was normal and apart from paleness of all the muscles and an impression that the musculature appeared wet, no specific areas of dystrophy were apparent except possibly in the fore-limb.

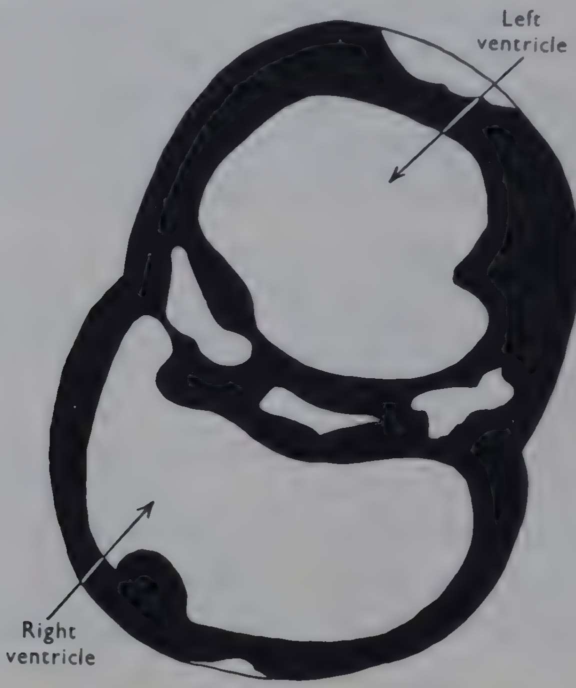

Fig. 2. Cross-section of the heart of calf no. AO.O. 4 at the level of the coronary pillars. White areas of wall represent dystrophic areas.

Calf no. CLO.E. 3. No abnormality of the viscera was observed save that both kidneys showed haemorrhagic infarcts. The heart muscles appeared quite normal. The musculature, on the other hand, was severely dystrophic. The diaphragm showed small areas of dystrophy and the adductor and brachial muscles appeared grossly affected throughout. This calf was the most severely affected of the group.

Calf no. CLO.E. 4. This calf, killed as a control for calf no. CLO.O. 4, was quite normal at post-mortem examination.

Group CLO.O. Cod-liver oil diet without α-tocopherol

Calf no. CLO.O. 1. The heart was dark in colour and extremely flabby. Myocardial degeneration could be seen in the internal ventricle walls. The brain showed an area of apparent inflammation in the right frontal lobe. Other internal organs were normal. The muscles were generally very pale and had a fibrous appearance. Obvious dystrophic areas were few.

Calf no. CLO.O. 2. The internal organs appeared quite normal. The heart appeared to be unaffected. The skeletal musculature, however, was grossly dystrophic. The occipital muscles of the neck were completely white, and the leg muscles were dystrophic throughout. Thus the supraspinatus showed intense white mottling, with oedema between the muscle bundles; the coraco-radialis was completely white, whereas the anterior extensor of the metacarpus and the semi-membranosus appeared normal.

Calf no. CLO.O. 3. The carcass was fairly fat and the fat showed a typical brown discoloration. There was a tendency for some gelatinous breakdown, especially at the rib insertions. The internal organs, including the heart, were normal. A gross involvement of the diaphragm was, however, present; this, in the absence of pneumonia, accounted for the severe dyspnoea observed before death. The skeletal muscles were very severely dystrophic; in fact the only groups of muscles not obviously affected were the facial muscles and the muscles running parallel to the trachea.

Calf no. CLO.O. 4. Some increase in pericardial fluid and pneumonia of the right apical lobe of the lung was noted. The heart was normal except for small haemorrhages on the auriculo-ventricular valve. Other internal organs were normal. The skeletal musculature was again grossly dystrophic, though not quite so seriously as in calf no. CLO.O. 3.

General observations

Several features of the above post-mortem examinations are of interest. The smell of the freshly killed carcasses was extremely characteristic. The carcasses of the dystrophic calves had a peculiar, offensive sweet odour, and most calves that had received cod-liver oil had a very slight fishy odour. Browning of the fat was apparent in two dystrophic carcasses only. The dystrophic lesions in the muscles were highly variable. In some muscles the dystrophic areas were highly circumscribed, and in others the areas ran from the origin to the insertion of the muscle, parallel to the line of the muscle bundles. In some muscles only the outer shell remained normal, whereas in others it was this area alone that was dystrophic. A photograph of grossly affected muscles is given in Pl. 2. This is a section through the left femur of one of the calves in the earlier series and shows lesions similar to those later observed.

Attempts were made to classify muscles and muscle groups according to the degree to which they were affected. It was quite obvious that from calf to calf there was no agreement either between the least affected or the most affected muscles. In one calf the supraspinatus was the most involved; in another, it was the least. On the other hand, when one side of the carcass was compared with the other, it was evident that there was a high degree of bilateral symmetry. It could not be concluded, in agreement with Hjärre & Lilleengen's (1936*a*, *b*) description of *weisses Fleisch*, that the lesions were mostly in the infra- and supraspinatus. Fig. 3 illustrates this symmetry, and also shows that there was no close association of the incidence of dystrophy with any particular muscle. The pattern of the visibly dystrophic lesions within each muscle, however, did not conform exactly with bilateral symmetry, but in general where a muscle was partly dystrophic, perhaps with 25 % involvement on the left side, the corresponding muscle was involved to the same extent on the right side.

In some instances it was possible to state that the front limb was more affected than the hind, or the shoulder muscles more than those of the arm or forearm. Pappenheimer (1940–1) has stated that in guinea-pigs and rabbits dystrophic lesions are not usually bilaterally symmetrical. Pamukcu (1948), however, has observed bilaterally symmetrical lesions in the former species. In the *weisses Fleisch* disease of

Hjärre & Lilleengen (1936*a*, *b*) lesions are symmetrical, and in human muscular dystrophy the disease generally affects both legs and both shoulders (Bicknell & Prescott, 1946). Attempts were then made to relate the severity with which a particular group of muscles was affected to the posture adopted by the calf before death. Agreement was good. Incidence of dystrophy in the rhomboideus muscle and to a lesser extent the trapezius was associated with the winged scapula of calf no. CLO.O. 3. The characteristic flexing of the carpus (knee) was associated with involvement of the shoulder muscles and those of the humerus group. Where spreading of the hoof claws was noted, the anterior extensor of the metacarpus and the muscles of the lower forearm appeared most affected. The adoption of a peculiar attitude and unnatural gait seemed related to removal of strain from these affected muscles. The calves in which the dystrophy was most marked in the hind-limbs minimized strain on these muscles by protrusion of the head and body and by placing the fore-limbs well under the body. This attitude is shown in calf no. CLO.O. 3 (Pl. 1, 3). Where dystrophy was most marked in the fore-limbs strain on these was minimized by a transference of weight to the hind-limbs and a forward stance, as is shown by the photograph of calf no. CLO.E. 2 (Pl. 1, 2).

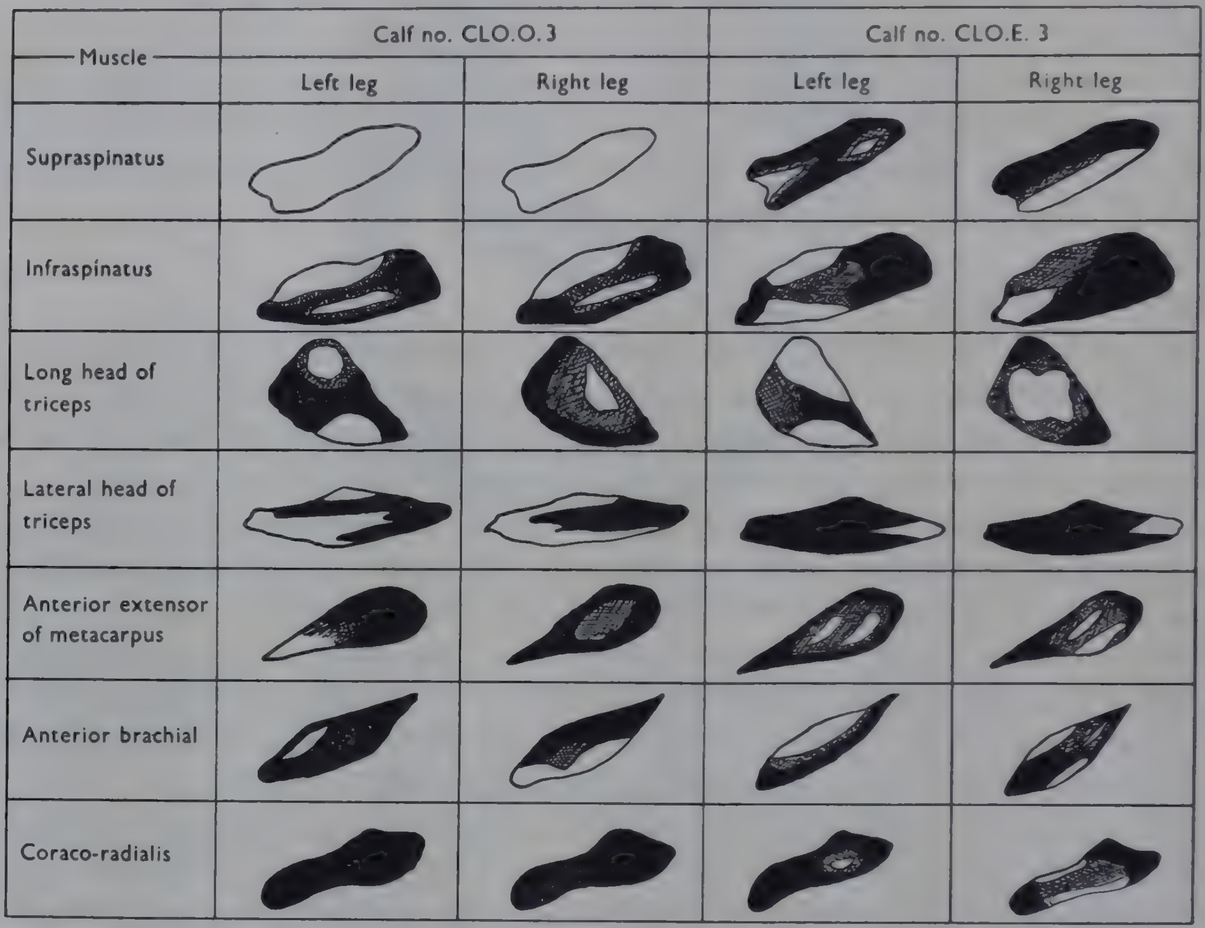
Muscle
Calf no. CLO.O.3
Calf no. CLO.E. 3
Left leg
Right leg
Left leg
Right leg
Supraspinatus
Infraspinatus
Long head of triceps
Lateral head of triceps
Anterior extensor of metacarpus
Anterior brachial
Coraco-radialis

Fig. 3. Lesions of the muscles of the right and left front limbs of calves nos. CLO.O. 3 and CLO.E. 3, illustrating the bilateral symmetry of the dystrophic lesions. White areas are dystrophic; cross-hatched areas are mildly affected; black areas are normal.

The presence of pneumonia in a proportion of the calves is of interest. Davis & Maynard (1938) noted that a high proportion of their calves given cod-liver oil showed pneumonia-like symptoms, though they did not succumb to any dystrophic disease. Most of the pneumonia encountered in the present study was probably caused by food entering the lungs, and this in turn was related to lack of control of the throat and tongue muscles.

Table 6. *Mean weights of different organs of the calves*

	Vitamins A and D supplied as			
	Cod-liver oil		Solution in arachis oil	
Organ	With vitamin E	Without vitamin E	With vitamin E	Without vitamin E
		(g)		
Brain	290	266	283	283
Liver	827	862	911	896
Heart	232	242	309	273
		(g/kg body-weight)		
Brain	8·47	7·51	6·47	6·80
Liver	24·2	24·3	20·9	21·5
Heart	6·78	6·85	7·07	6·54

Weights of the major organs of the calves were determined and are summarized in Table 6. No differences of a statistical significance were present. If the calves were classified according to the presence or absence of dystrophy (see Table 2), there was no evidence of any hypertrophy or atrophy of these organs in affected animals. The hearts of those animals that had been found abnormally flabby at post-mortem (calves nos. CLO.O. 1 and CLO.E. 1), however, tended to be the heaviest. The testes of the animals appeared to be of normal weight and no difference that could be ascribed to difference in treatment was seen.

DISCUSSION

The calves that were given the arachis-oil diet with a supplement of α-tocopherol appeared normal both before and at post-mortem examination. Those calves that received the same diet but did not receive α-tocopherol, however, were abnormal in behaviour and carriage, and when slaughtered three were found to have grossly dystrophic skeletal muscles and the other a degenerate myocardium. From the data presented it would seem that the lesions observed and the clinical appearance of the animals were in fact due to a dietary deficiency of vitamin E, thus confirming the preliminary observations. The disease appears quite comparable to the 'white muscle disease' of Vawter & Records (1947), the *weisses Fleisch* of Hjärre & Lilleengen (1936*a*, *b*) and the waxy degeneration (*wachsartige Muskeldegeneration*) of earlier authors. These diseases were not experimentally produced but were found in animals on farms. We have found and confirmed two similar field cases in Ayrshire. The lesions that have been described in the calves appear to be quite comparable to those described in other animals. They are bilaterally symmetrical and are not limited to particular muscles; they are sometimes associated with haemorrhages, and render the muscle non-translucent. The signs, too, show considerable agreement—somnolence and unwillingness to place particular muscles under stress, unnatural postures and, finally, complete prostration.

Of the eight calves that were given cod-liver oil with or without vitamin E, only one did not show signs of dystrophy and only one was not dystrophic on post-mortem examination. The signs and post-mortem appearances of these animals were in all respects similar to those of the calves given the vitamin-E-deficient diet in which fat-soluble vitamins were supplied in solution in arachis oil. It may be concluded, therefore, that administration of cod-liver oil results in a severe exacerbation of symptoms of dystrophy and effectively neutralizes the protective action of α-tocopherol. As little as 15–18 ml. cod-liver oil given daily was sufficient to render ineffective the protective action of a daily supplement of 50 mg DL-α-tocopheryl acetate. It may also be concluded, therefore, that the syndrome of cod-liver oil poisoning of calves described by the Scandinavian workers represents a conditioned vitamin E deficiency.

The length of time taken for the condition to develop is of interest. In severe cases the calves were first noted to be abnormal at 21–41 days from the time the experimental diet was first given. Two very severe cases were first noted at 24 and 43 days. Such variation shows that individuals differ considerably in their sensitivity to vitamin E deficiency. Whether this is due to variation in body stores of tocopherols, to differences in daily requirement or to other causes is not known.

SUMMARY

1. Observations on a disease of young calves are given and the reasons for the diagnosis of vitamin E deficiency are presented.

2. An experiment with sixteen Ayrshire calves is described. All were given a basal diet containing dried skim milk, lard and a mineral supplement. Eight were given vitamins A and D as the pure substances dissolved in arachis oil, and eight were given equivalent amounts as cod-liver oil. Four animals in each of these groups received in addition 50 mg DL-α-tocopheryl acetate daily.

3. Signs of dystrophy occurred in all groups other than that receiving both α-tocopherol and the arachis-oil solution. These signs are described in detail.

4. Nitrogen-balance studies with four calves showed that animals that were found dystrophic at post-mortem stored less nitrogen than the others. The high urinary excretion of nitrogen was due to an increase in excretion of urea and ammonia.

5. The oxygen consumption of dystrophic animals was increased. The excessive nitrogen katabolism and the increased heat production were probably interrelated.

6. Post-mortem examination of the affected calves revealed an extensive dystrophy with oedema, involving both skeletal and cardiac muscles. The dystrophy was bilaterally symmetrical.

7. The characteristic attitudes adopted by the calves before death, flexing of the carpus, straightening of the hind-limbs, spreading of the claws of the foot and winging of the scapulas, were all associated with severe dystrophy of the muscles controlling the movement of the joints concerned.

8. The daily ingestion of 15–18 ml. cod-liver oil neutralized the protective action of 50 mg DL-α-tocopheryl acetate daily.

9. The abnormalities found are compared with those described in similar naturally

K. L. BLAXTER, P. S. WATTS AND W. A. WOOD. NUTRITION OF THE AYRSHIRE CALF. 8 *Plate* 1

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2

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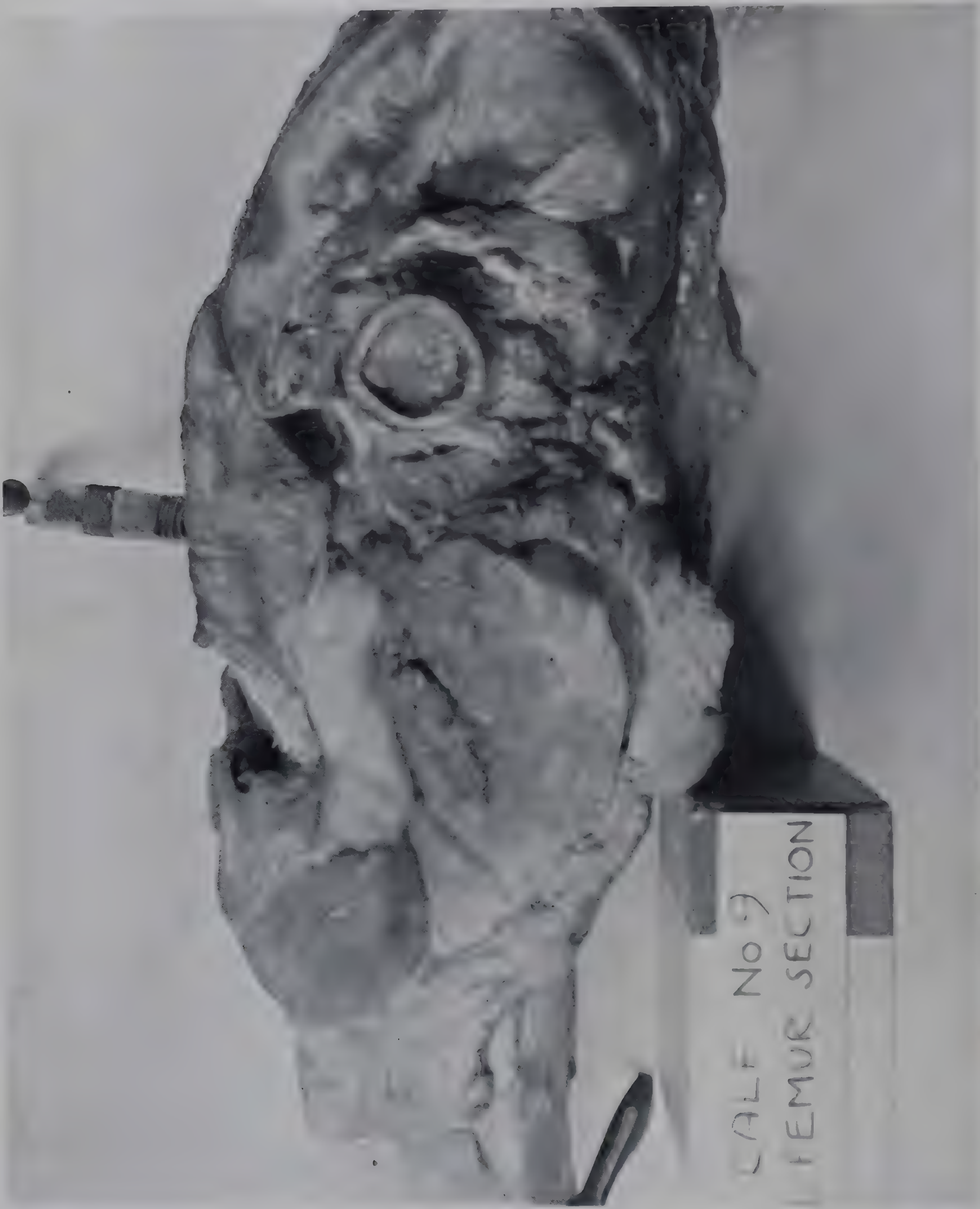
CALF No 9
FEMUR SECTION

occurring diseases in Europe and America, and it is concluded that all these conditions probably result from vitamin E deficiency.

We are indebted to Dr T. Moore for the gift of α-tocopheryl phosphate for use in the preliminary trials, and to Miss A. M. Goodall for the histological examination of tissues from two of the calves in the earlier series. Our thanks are due to Mr D. Paterson, Mrs A. Arbuckle and Miss E. Semple for their technical assistance.

EXPLANATION OF PLATES

Plate 1

1. Normal calf no. AO.E. 3, which received sufficient vitamin E and showed no signs of dystrophy.
2. Dystrophic calf no. CLO.E. 2. Dystrophy was most evident in the fore-limbs and the strain on these muscles was minimized by transference of weight to the hind-limbs.
3. Dystrophic calf no. CLO.O. 3. Dystrophy was most evident in the hind-limbs, resulting in the abnormal stance adopted.
4. Shoulders of dystrophic calf no. AO.O. 4, showing protrusion of the suprascapula above the level of the spinal processes. This sign is comparable to winged scapula in muscular dystrophy of man.

Plate 2

Cross-section through the femur of a dystrophic calf, showing large white areas of dystrophy interspersed with small areas apparently unaffected.

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The Nutrition of the Young Ayrshire Calf

9. Composition of the Tissues of Normal and Dystrophic Calves

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(*Received* 27 *March* 1951)

In a previous paper (Blaxter, Watts & Wood, 1952) data were presented which showed that experimental diets could be prepared which, when given to young calves, caused a very high incidence of muscular dystrophy. The present paper is concerned with the composition of muscles and other tissues of these calves.

EXPERIMENTAL

General

The plan of the experiment, details of experimental animals, their diets and their management were given in the previous paper (Blaxter *et al.* 1952). The same nomenclature is used here, namely:

Group AO.E. Vitamins A and D were supplied in solution in arachis oil; 50 mg α-tocopheryl acetate were given daily.

Group AO.O. Vitamins A and D were supplied in solution in arachis oil; no vitamin E was given.

Group CLO.E. Vitamins A and D were supplied by cod-liver oil; 50 mg α-tocopheryl acetate were given daily.

Group CLO.O. Vitamins A and D were supplied by cod-liver oil; no vitamin E was given.

Individual calves are referred to by treatment and replication number.

Methods

Post-mortem methods and details of dissections have been described (Blaxter *et al.* 1952). The following muscles were analysed chemically: supraspinatus, infraspinatus, long head of triceps, lateral head of triceps, anterior brachial, coraco-radialis, anterior extensor of the metacarpus, biceps femoris, semi-tendinosus, semi-membranosus, rectus femoris, vastus medialis, adductor, gastrocnemius, diaphragm, tongue and heart. Brain and liver were also analysed, but not in such great detail. All tissues were dissected free of tendon and fascia and were macerated in a high-speed blendor. On this fresh material water was estimated by direct drying at 100°; cholesterol and cholesteryl esters by the method of Schoenheimer & Sperry (1934) as modified by Sobel & Meyer (1945); nitrogen by Kjeldahl; acid-soluble, lipid and nucleic-acid phosphorus compounds were separated by the method of Schneider (1945) and the phosphorus was determined by the method of Fiske & Subbarow (1925), creatine by the method of Rose, Helmer & Chanutin (1927) and collagen by the method of Spencer, Morgulis & Wilder (1937). Non-protein and total-globulin nitrogen were determined on isotonic potassium chloride-bicarbonate extracts of the fresh muscle prepared by maceration in a high-speed blendor using tungstic acid as a precipitant. Myosin was estimated through the nitrogen content of the precipitate formed after dilution of the buffer extracts at the iso-electric point of myosin. Stroma proteins were estimated by determination of the nitrogen content of the washed residuum of the extract. On the dried muscle and tissue, fat was determined by extraction with diethyl ether and light petroleum; calcium and magnesium by the method of McCrudden (1911–12), iron by the method of Woiwod (1947), sodium by the method of Butler & Tuthill (1931) and total phosphorus by the method of Fiske & Subbarow (1925). Potassium was determined by direct weighing of the cobaltinitrite precipitate (Rowland, 1948).

RESULTS

Dry-matter content of the muscles

The mean dry-matter content of the muscles is shown in Table 1. The frequency distributions presented in Fig. 1 show that the dry-matter content of the muscle tissues decreased, and conversely the water content increased, when the animals were given cod-liver oil as a source of vitamins A and D and also when vitamin E was excluded from the diet. Analysis of variance of the results of Table 1 showed that both the effects of cod-liver oil and lack of vitamin E in increasing the water content of the muscle were significant at $P=0{\cdot}05$. There was no significant interaction or synergistic effect between presence of cod-liver oil and absence of α-tocopherol. In general, the fore-limbs tended to have a higher water content than the hind. This was not always true, for in some calves where a hind-limb was grossly dystrophic it had the higher water content.

From the notes made when the calves were slaughtered, the various muscles were classified according to the degree to which they showed dystrophic involvement. Normal muscles were classified in grade 1, and severely affected muscles in grade 5. The mean dry-matter content of muscles in each grade is shown in Table 2. The dystrophic muscles of severely affected calves contained more water than the normal muscles of the same calf. Even those muscles that appeared quite normal contained, however, less dry matter and more water than those of calves that were both clinically normal and normal at post-mortem examination.

The decrease in dry-matter content and concomitant increase in the water content of dystrophic skeletal muscle did not suggest that the condition was comparable to human muscular dystrophy or the muscular dystrophy of the rabbit, where

Table 1. *Mean dry-matter content of muscles from sixteen calves, four in each group, given different diets*

	Vitamins A and D supplied by			
	Cod-liver oil		Solution in arachis oil	
Muscle	Without vitamin E (%)	With vitamin E (%)	Without vitamin E (%)	With vitamin E (%)
Supraspinatus	18·3	19·2	20·1	21·0
Infraspinatus	18·6	19·5	20·0	21·0
Long head of triceps	18·2	20·8	19·8	21·4
Lateral head of triceps	18·3	19·5	20·1	20·7
Anterior brachial	19·3	20·2	21·0	21·8
Coraco-radialis	19·2	20·3	19·8	20·3
Anterior extensor of metacarpus	21·2	20·9	19·7	21·1
Mean for anterior limb	18·99	20·06	20·06	21·05
Biceps femoris	19·9	19·8	20·9	21·7
Semi-tendinosus	21·5	20·9	21·3	21·9
Semi-membranosus	19·6	21·3	20·7	21·6
Rectus femoris	19·2	19·9	20·4	21·8
Vastus medialis	18·9	21·4	20·3	21·3
Adductor	19·3	19·7	20·7	21·0
Gastrocnemius	18·9	20·6	20·3	21·3
Mean for posterior limb	19·60	20·52	20·64	21·51
Mean for both limbs	19·29	20·29	20·35	21·28
Heart	20·05	20·03	19·8	19·9
Diaphragm	17·2	19·4	20·7	20·8
Tongue	21·4	21·4	22·6	25·0

Table 2. *Mean dry-matter content of anterior-limb muscles from severely affected and mildly affected calves classified according to the involvement of the muscle*

Involvement of muscle		Severely affected calves		Mildly affected calves	
Extent	Grade*	No. of muscles examined	Dry matter (%)	No. of muscles examined	Dry matter (%)
Completely white throughout	5	4	18·10	0	—
Large white areas involving over 50 % of the muscle substance	4	4	19·02	0	—
Scattered small white areas or single white areas involving less than 50 % of the muscle substance	3	6	19·53	7	20·13
Generally pale or fibrous in appearance: no specific white areas	2	4	19·83	4	20·20
Apparently normal muscle†	1	3	20·53	10	20·39

* The grading was from 1 for a normal muscle to 5 for a severely affected one.

† The dry-matter content of the normal muscle from anterior limbs of normal calves was 21·05 % (mean of twenty-eight determinations).

an increase in fat deposition must imply a marked fall in water content. This is well illustrated by the results of Goettsch & Brown (1932). The triceps of normal rabbits contained only 1·1 % fat and 76·5 % water. The dystrophic triceps contained 34·5 % fat and 50·1 % water. With rats, in which dystrophy is not accompanied by extensive fat deposition, Knowlton & Hines (1938) and Knowlton, Hines & Brinkhous (1939) showed that an increase occurred in water content from 75·4 to 76·6 %. Table 1 shows the results of analyses of diaphragm and tongue muscles of the calves and indicates no major differences. Differences in the dry-matter content of the heart muscle of the calves were not statistically significant, but one high value was recorded for calf no. CLO.O. 3. The dry-matter content of the heart muscle of those animals in which evidence of abnormality had been found did not differ significantly from the mean value for their particular group.

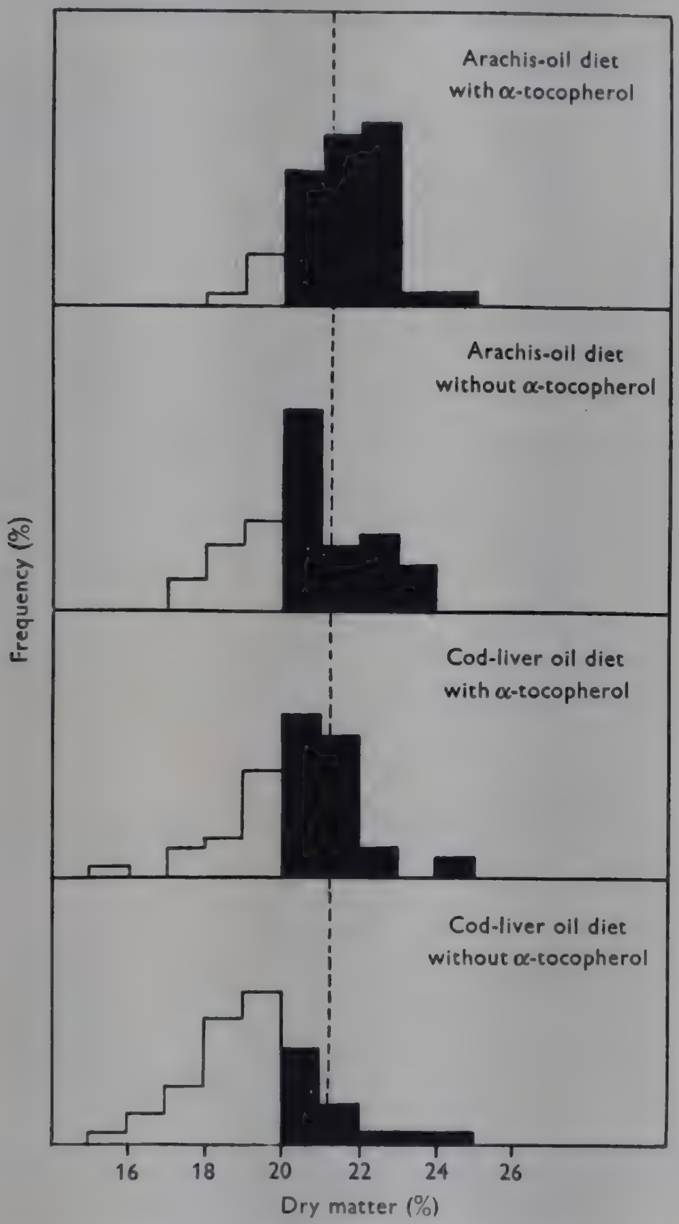

Fig. 1. Percentage frequency-distributions of the dry-matter content of the muscles of calves. The dotted line is the mean content of group AO.E. Fifty-six skeletal muscles were included in each group. Black areas represent muscles containing more than 20 % dry matter, a quantity which may be regarded provisionally as the lower limit of the normal.

Ash content of the muscles

The ash content of sixteen muscles of two animals in each group was determined. Mean results are given in Table 3.

Table 3. *Mean values for two calves in each group for the ash content of the dry matter of muscles*

	Vitamins A and D supplied as			
	Cod-liver oil		Solution in arachis oil	
Muscle	Without vitamin E (%)	With vitamin E (%)	Without vitamin E (%)	With vitamin E (%)
Anterior limb (seven)	7·18	5·64	5·28	5·02
Posterior limb (seven)	6·94	5·63	4·94	5·28
All limb muscles	7·06	5·63	5·11	5·15
Diaphragm	9·74	5·06	4·16	4·43
Tongue	4·35	4·92	3·38	3·04

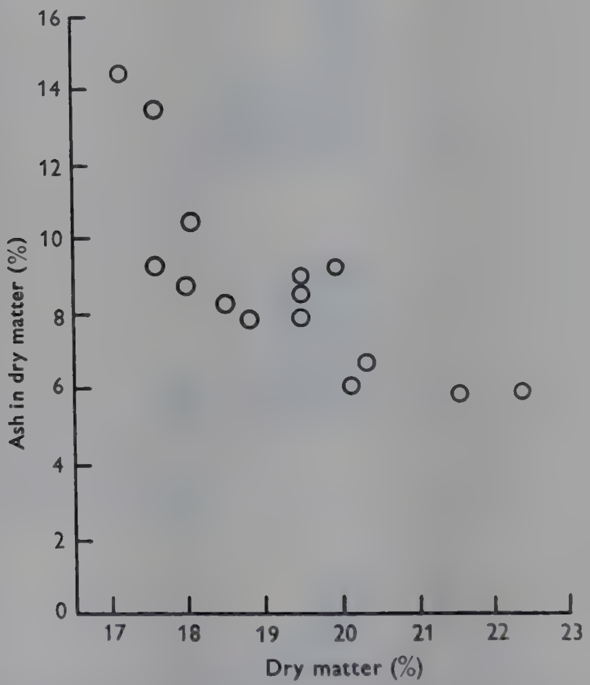

Fig. 2. Relation between the dry-matter content and the ash content of the dystrophic muscles of calf no. CLO.O. 3.

Although the number of observations was small, statistical analysis indicated that the differences between treatment means were significant at odds of 1 : 14. The concordance of the data suggest that dystrophic animals had muscles with a considerably higher ash content. Thus the highest ash content recorded for a skeletal muscle was 13·3 % for the supraspinatus muscle of calf no. CLO.O. 3, this muscle being a completely white muscle. The diaphragm in this animal contained 14·4 % ash. In Fig. 2 the ash content of all the muscles of this calf is plotted against their dry-matter content. Where the water content of the muscle was high, the ash content of the dry matter was also high. This suggests that infiltration of electrolytes into the muscle accompanied the water infiltration.

Analyses were then made on a number of dystrophic and normal muscles to find what minerals were present in excess in the grossly dystrophic muscles. Table 4 shows that the magnesium and phosphorus content of the muscles that were severely affected and had a high ash content remained in the normal range, but the calcium content of severely affected muscle was nearly ten times as great as in normal muscle. This content does not represent a severe calcification, but nevertheless shows that excessive calcium deposition had occurred. Marked calcification of the muscles was demonstrated in dystrophic rabbits by Fenn & Goettsch (1937) and by Morgulis & Osheroff (1938). Taking their results together, it appears that in animals given diets that induced dystrophy but slowly, the calcification was the most severe. In the present calves the dystrophy may be regarded as of a more acute nature, and the absence of extreme calcification supports the contention that only in chronic dystrophy does calcification become severe.

Table 4. *Calcium, magnesium and phosphorus content of selected muscles of two calves, expressed on dry weight*

Calf no.	Muscle	Dystrophic lesions	Ash (%)	Ca (mg/100 g)	Mg (mg/100 g)	P (%)
CLO.O. 4	Biceps femoris	Whole muscle white	7·99	202·8	80·0	1·08
	Semi-membranosus	Muscle very pale	6·48	57·3	94·9	1·09
	Heart	Normal	5·18	30·8	102·2	1·08
CLO.E. 4	Semi-membranosus	Normal	5·12	28·1	101·0	0·96
	Biceps femoris	Normal	5·11	24·8	80·7	1·02
	Heart	Normal	5·38	26·7	94·2	1·25

Table 5. *Mean water content and mean sodium, potassium and creatine content of normal and dystrophic muscles of calves, expressed on dry weight*

Muscle	No. of samples	Water (%)	Creatine	Na	K	Na	K	Total Na+K
			(mg/100 g)			(mg equivalents/l. muscle water)		
'Normal'* from calves in group AO.E.	4	77·9	451	—	1917	—	134·8	—
'Normal'* from affected calves	5	78·1	445	30·5	1721	38·5	123·2	161·7
Obviously dystrophic muscle	4	80·6	289	55·2	1612	55·9	98·6	154·5
Single very severely affected muscle†	1	85·0	59	124·0	919	95·1	41·5	136·6

* 'Normal' refers to muscle that was classified at post-mortem examination as completely free from visible dystrophic ions.

† This muscle was one taken from an animal given in an unpublished experiment a diet comparable to that containing d-liver oil. Unfortunately all comparable samples from the present series had been used when the sodium and potassium alyses were made. This muscle was comparable to the coraco-radialis of calf no. CLO.O. 2.

Sodium and potassium were determined on a series of muscles, and the mean results are given in Table 5. The dry matter of dystrophic muscle contained more sodium and less potassium than that of normal muscle. The water content of the dystrophic muscle was increased. Expressed on a water basis, the number of mg equivalents present as sodium increased largely at the expense of potassium, and there was an indication of a decrease in the total number of mg equivalents of sodium and potassium in the most affected muscle. These results show that major changes occurred in the distribution of water in the muscle tissues and can be interpreted as an increase in extracellular fluid and a decrease in intracellular fluid. Increases in sodium content and decreases in potassium content have been demonstrated in dystrophic rabbit muscle by Morgulis & Osheroff (1938). It may be concluded, therefore, that with the calf, as with the rabbit, muscular dystrophy is associated with an increase in mineral content of muscle, as a result of both a mild pathological calcification and a change in the proportions of intra- and extracellular fluids of the tissue.

Total lipid content of the muscles

The mean total lipid contents of the dried muscles are shown in Table 6. There were no differences between treatments that could be judged significant. Owing to the highly variable incidence of dystrophy in the entire musculature, these muscles, however, were not necessarily representative of the extent of dystrophy present in the whole carcass, and, in the second half of the experiment, two muscles were taken from each animal to ascertain if the more severely affected muscle differed in total lipid content from the less affected one. In the six animals that showed dystrophic lesions, the more severely affected muscle had a mean lipid content of 3·62 %, whereas the less severely affected contained 2·45 %, and where the difference in the extent of dystrophy was the greatest, the difference in lipid content was also the greatest. The twenty-four determinations comprising all the lipid analyses (eight in the first half of the experiment and sixteen in the second) were therefore grouped according to the creatine content of the muscle (assuming creatine content to be the most reliable index of dystrophic involvement), and the mean results are given in Fig. 3. It was apparent that the most severely affected muscle contained the most lipid. This is in accordance with the histological evidence presented for the guinea-pig and the rat (Goettsch & Pappenheimer, 1931; Pappenheimer, 1948), but the dystrophy found could hardly be classified as a lipodystrophy, since the content of lipid, though increased, was nevertheless very small.

Table 6. *Mean content of total lipid (ether extract) of the dry matter of skeletal muscle* and heart from four calves in each group*

	Mean total lipid (dry basis, %)	
Treatment	Skeletal muscle	Heart
Cod-liver oil diet without vitamin E	3·36	11·31
Cod-liver oil diet with vitamin E	3·65	11·77
Arachis-oil diet without vitamin E	4·96	11·57
Arachis-oil diet with vitamin E	2·87	11·64
Standard error of means	±0·74	±1·31

* The rectus femoris was analysed in each animal except calves nos. CLO.O. 3 and CLO.E. 3 where the semi-membranosus was taken.

Slight browning of the fatty deposits in certain dystrophic animals was noted, and this fat gave a brown fluorescence in ultraviolet light comparable to that found by Martin & Moore (1939) and by Moore & Wang (1943). No extraction of the abnormal pigment was made.

Cholesterol content of muscles

The total, free and esterified cholesterol was determined on fresh samples of the same muscles that were later analysed for total lipid. The mean results are given in Table 7. Statistical analysis showed that the effect of deprivation of vitamin E was to increase the total cholesterol ($P=0.05$) and the free cholesterol ($P=0.025$). The effect of inclusion of cod-liver oil was not statistically significant, but in only one instance did the muscle cholesterol content of the calves given cod-liver oil with vitamin E appear lower than that in those given arachis oil with vitamin E. That the cholesterol content of the dry matter of the muscle was increased in dystrophy was clearly shown by a classification of two muscles from each of six dystrophic calves, similar to that previously made for total lipid content. The results are shown in Table 8.

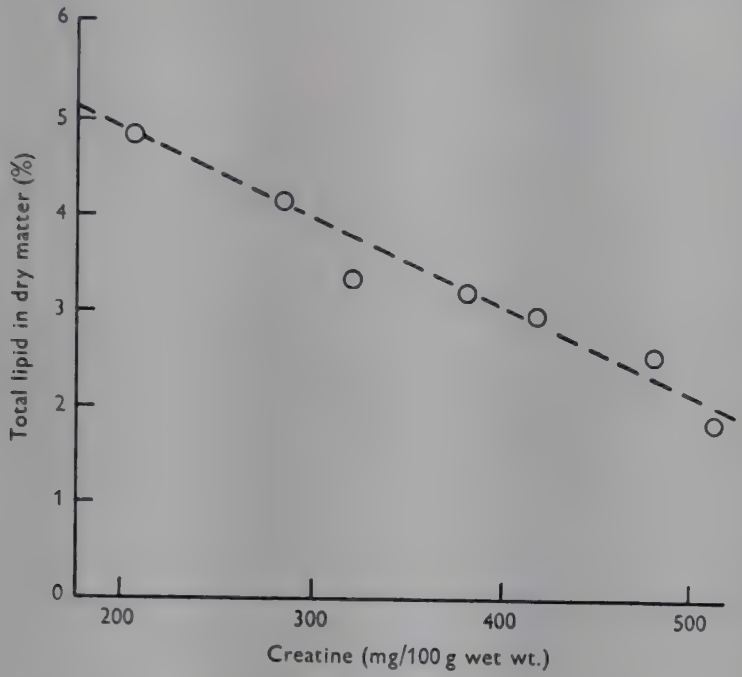

Fig. 3. Relation between the creatine content and the total lipid content of the muscles. Each point represents the mean result of simultaneous determinations of lipid and creatine content of either three or four muscles. The classification is based on creatine content. Muscles from all sixteen calves are included.

Table 8 shows that the most dystrophic muscles contained the most cholesterol. This appeared to be due to a large increase in free cholesterol rather than to an

Table 7. *Total, free and ester cholesterol content of skeletal muscle dry-matter from four calves in each group*

Treatment	Cholesterol (mg/100 g dry muscle) Total	Free	Ester
Cod-liver oil diet without vitamin E	718	645	73
Cod-liver oil diet with vitamin E	512	438	74
Arachis-oil diet without vitamin E	588	566	22
Arachis-oil diet with vitamin E	430	371	59
Standard error of means	±93	±83	—

Table 8. *Mean lipid, cholesterol and cholesteryl-ester content of dystrophic muscles—pair comparisons on each of six dystrophic calves*

(mg/100 g dry weight)

Muscle	Lipid	Cholesterol			Total cholesterol as percentage of fat
		Total	Free	Ester	
Most affected	3620	666	604	62	18·4
Least affected	2450	555	419	136	22·6
Normal*	2280	455	398	57	19·9

* Total for the comparable two muscles from each of two normal calves in the second half of the experiment.

increase in esterified cholesterol. The total cholesterol content of the fat did not change during dystrophy, so that it may be concluded that the observed increase in fat content was not solely due to an increase in cholesterol. This is not in agreement with the results obtained in dystrophic rabbits where the cholesterol content of the muscle was increased more than the total lipid content (Morgulis, Wilder, Spencer & Eppstein, 1938). More recent work (Heinrich & Mattill, 1943), however, has shown that with rats, even in the most severe dystrophy, the proportion of cholesterol in the total lipids increased but slightly and this change could not be judged significant. The results of Morgulis & Spencer (1936) with rabbits, though demonstrating a large change in cholesterol content, do not provide information on this aspect. In Fig. 4 the results of the analyses of skeletal muscles for cholesterol are plotted against those for muscle creatine. Dystrophic muscles were clearly lower in creatine and higher in cholesterol content.

The cholesterol content of the heart muscle was determined and the results are given in Table 9. There were no differences that could be ascribed to treatment effects.

Table 9. *Mean cholesterol content of heart muscles of sixteen calves, expressed on a dry basis*

(mg/100 g dry matter)

Treatment	Cholesterol		
	Total	Free	Ester
Cod-liver oil diet without vitamin E	673	661	12
Cod-liver oil diet with vitamin E	678	655	23
Arachis-oil diet without vitamin E	715	700	15
Arachis-oil diet with vitamin E	811	752	68

The hearts of calves nos. CLO.O. 1 and CLO.E. 1, which were flabby at post-mortem, contained 670 and 715 mg free cholesterol/100 g dry matter. The dystrophic heart of calf no. AO.O. 4 contained 665 mg free cholesterol/100 g dry matter. All these values are within the normal range. In rabbits suffering from severe dystrophy of the skeletal musculature Morgulis & Spencer (1936) also found the heart cholesterol content normal. In rabbits it is rare to find dystrophic cardiac musculature, even though the skeletal musculature is completely affected (Goettsch & Pappenheimer, 1931).

Nitrogen content of the muscles

The total nitrogen content of three muscles from the anterior limb, three muscles from the posterior limb and the ventricles of the heart was determined in every calf. Mean results are given in Table 10. Analysis of variance of the data given in Table 10 showed that the effect of vitamin E administration on the muscle nitrogen was not significant, and the effect of cod-liver oil in reducing the nitrogen content was only significant at odds of 13 : 1. Nevertheless, it was suggestive that those muscles that were the most dystrophic contained the least nitrogen. The muscles were therefore classified according to extent of dystrophy as shown in Table 11. The nitrogen content of the most affected muscles was much reduced, and they contained 2·48 g nitrogen/100 g dry matter less than the normal muscles. This difference was highly significant statistically.

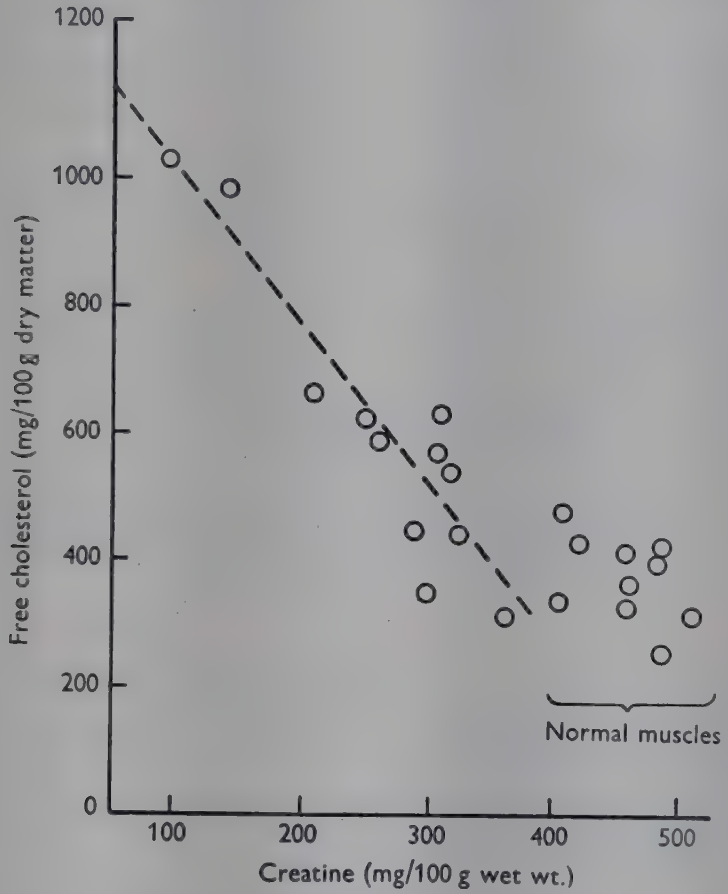

Fig. 4. Relation between the creatine content and the free cholesterol content of the muscles. Each point represents the result of a single determination of the creatine content and free cholesterol content of a skeletal muscle. Muscles from all sixteen calves are included.

Eight muscles, four from normal calves and four affected muscles from dystrophic calves, were analysed for stroma protein, non-protein nitrogen and total globulin nitrogen. The percentage of the total nitrogen present as stroma proteins was increased from 22·1 to 32·9 in the dystrophic muscle. This increase was highly significant ($P < 0\cdot001$). Conversely, the content of total globulin nitrogen declined. The percentage of non-protein nitrogen was not significantly affected. The decrease

Table 10. *Mean nitrogen content of skeletal and cardiac muscle of sixteen calves, four in each group, expressed on a dry basis*

	Vitamins A and D supplied by			
	Cod-liver oil		Solution in arachis oil	
Muscle	Without vitamin E (%)	With vitamin E (%)	Without vitamin E (%)	With vitamin E (%)
Supraspinatus	14·76	14·11	14·58	14·53
Infraspinatus	13·94	14·07	14·83	14·38
Long head of triceps	13·90	14·13	14·37	14·66
Mean for anterior limb	14·20	14·10	14·59	14·52
Semi-membranosus	14·30	13·95	14·52	14·90
Rectus femoris	14·54	14·31	14·69	14·69
Biceps femoris	12·78	12·89	14·00	14·74
Mean for posterior limb	13·87	13·72	14·40	14·78
Mean for both limbs*	14·04 ± 0·47	13·91 ± 0·47	14·50 ± 0·47	14·65 ± 0·47
Heart	12·85	13·30	13·47	13·54

* Value with its standard error.

Table 11. *The nitrogen content of the muscles of the sixteen calves classified according to the involvement of the muscle*

Involvement of muscle	Grade*	No. of muscles examined	Mean nitrogen content of muscle† (%)
Completely white throughout	5	5	11·90 ± 0·42
Large white areas involving over 50 % of the muscle substance	4	9	13·83 ± 0·31
Scattered small white areas or single white areas involving less than 50 % of muscle substance	3	13	14·26 ± 0·26
Generally pale or fibrous in appearance with no specific white areas	2	8	14·06 ± 0·33
Apparently normal	1	11	14·38 ± 0·28

* For definition see Table 2. † Value with its standard error.

in the colour of the dystrophic muscles suggested a decrease in the amount of muscle haemoglobin in the globulin fraction. Iron determinations in a series of muscles are given in Table 12. The classification of the dystrophic muscles was based on their gross appearance. It will be noted that the affected muscles contained less iron than the normal muscles. This difference was significant at $P = 0·05$. The decline in the total iron of the muscles reflects a decline in muscle haemoglobin, since the amount of muscle iron present as haemosiderin, phosphocarnic acid and in other phosphate combinations is presumably small.

A further indication of the marked changes in the protein distribution of the muscles is to be found in their collagen content. The collagen-nitrogen content of normal muscles was 1·8–1·9 % on a fresh basis. In severely affected muscles the content was increased up to 2·6 %. The most severely affected muscle that was analysed, the biceps

Table 12. *Mean values for total creatine content and iron content of muscles*

	Normal* muscle from normal calves in group AO.E.	Muscle from affected calves	
		Slightly affected	Severely affected
No. of samples	5	3	3
Creatine content (mg/100 g wet muscle)	428	318	187
Iron content (mg/100 g dry muscle)	3·85	3·48	2·60

* 'Normal' refers to muscle that was classified at post-mortem examination as completely free from visible dystrophic lesions.

femoris of calf no. CLO.O. 4, containing only 141 mg creatine/100 g fresh weight, had a collagen-nitrogen content of 3·5 %, which is nearly double that found in normal muscles from normal calves.

It would appear, therefore, that large changes occur in the protein fraction of the muscles, comprising a marked increase in the amount of collagen and stroma proteins, a slight fall in the non-protein nitrogen fraction and a marked decrease in the content of globulins, which, on the basis of iron determinations, appears to be due in part to a fall in the content of muscle haemoglobin. The latter would account for the white colour of the affected musculature.

Creatine content of the muscles

The creatine content of the main skeletal muscles is given in Table 13, and Fig. 5 expresses the results in the form of frequency distributions. These show that a marked decline in the creatine content of the muscles occurred both when the calves that received the arachis-oil diet were deprived of vitamin E and when cod-liver oil was substituted for arachis oil. An even greater fall in the creatine content of the muscles occurred when the animals that received cod-liver oil were deprived of vitamin E. Analysis of variance of the results given in Table 13 showed that both the depressing effect of vitamin E deprivation and that of cod-liver oil administration on muscle creatine were significant ($P=0·05$). These results are in complete agreement with those of Goettsch & Brown (1932) and Morgulis & Spencer (1936) with rabbits and also with those of Shimotori, Emerson & Evans (1940) with guinea-pigs and of Knowlton & Hines (1938) with rats.

Closer examination of Table 13 shows that not all muscles were affected to the same extent. This was already evident at post-mortem examination (Blaxter *et al.* 1952), and Table 14 summarizes typical results for calf no. CLO.E. 3 and calf no. CLO.O. 3.

From Table 14 it can be seen that the supraspinatus muscle of calf no. CLO.O. 3 contained less creatine than the same muscle of calf no. CLO.E. 3; the coraco-radialis contained approximately the same amount in both calves, whereas the anterior extensor of the metacarpus of calf no. CLO.O. 3 contained more creatine than that of calf no. CLO.E. 3. A high degree of bilateral symmetry may also be inferred. Fig. 6 illustrates this symmetry. The correlation between the creatine content of muscles on the right side and those on the left was +0·905, which was highly significant statistically. It was quite evident, however, that though quite symmetrical in the individual, the dystrophic lesions were not limited to particular muscles in different individuals.

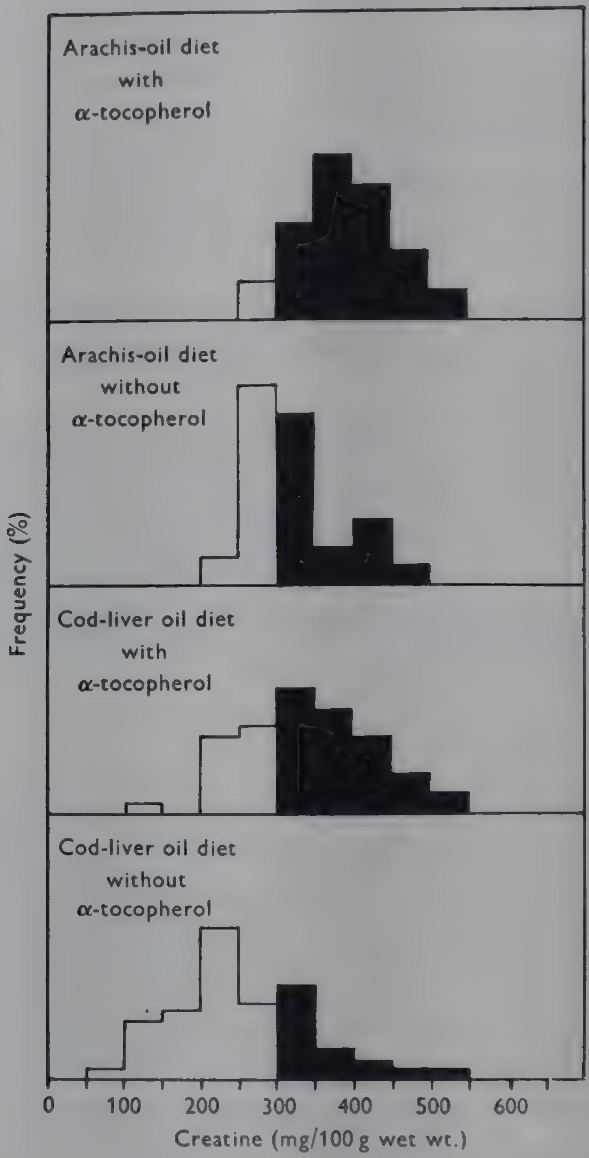

Fig. 5. Percentage frequency-distributions of the creatine content of skeletal muscles of calves (fifty-six muscles in each group). Black areas represent muscles with a creatine content greater than 300 mg/100 g, which may be classified as normal; white areas represent muscles with a creatine content less than 300 mg/100 g, which may be classified as abnormal.

In Fig. 7 frequency distributions are given which illustrate the accuracy of the visual classification of the muscles from dystrophic calves as far as prediction of their creatine content was concerned. There was general agreement, but the visual classification was by no means exact and there was considerable overlapping. Part of this was due to inexperience in visual classification, for, with calves that died later in the experiment, prediction was extremely good. The lowest creatine content of an affected muscle was 96 mg/100 g for the completely dystrophic coraco-radialis of calf no. CLO.O. 2.

Table 13. *Mean creatine content of muscles from sixteen calves, four in each group*

(mg/100 g fresh weight)

	Vitamins A and D supplied by			
	Cod-liver oil		Solution in arachis oil	
Muscle	Without vitamin E	With vitamin E	Without vitamin E	With vitamin E
Supraspinatus	182	283	281	357
Infraspinatus	213	271	293	362
Long head of triceps	221	342	304	391
Lateral head of triceps	200	261	288	343
Anterior brachial	258	291	287	378
Coraco-radialis	360	387	374	366
Anterior extensor of metacarpus	385	347	283	386
Mean for anterior limb	259·9	311·6	301·4	369·0
Biceps femoris	206	350	333	441
Semi-tendinosus	256	378	373	433
Semi-membranosus	350	442	372	443
Rectus femoris	302	371	363	443
Vastus medialis	223	338	315	387
Adductor	261	374	378	368
Gastrocnemius	209	316	279	383
Mean for posterior limb	258·1	367·0	344·7	414·0
Mean for both limbs	259·0	339·3	323·0	391·5
Heart	239	229	226	256
Diaphragm	141	233	263	313
Tongue	176	219	221	239

Table 14. *Creatine content of muscles from both sides of the bodies of calves nos. CLO.E. 3 and CLO.O. 3*

(mg/100 g fresh muscle)

	Calf no. CLO.E. 3		Calf no. CLO.O. 3	
Muscle	Right side	Left side	Right side	Left side
Supraspinatus	304	261	99	105
Coraco-radialis	504	516	531	511
Anterior extensor of metacarpus	323	314	472	513

Distribution of phosphorus compounds in the muscles

Table 15 summarizes the results obtained by partition of the total phosphorus. A small and variable quantity of 'protein phosphorus' was ignored in the transformation of the analytical data to percentages. Analysis of variance of the results of Table 15 showed that the differences between treatments were not statistically significant. This conclusion, however, did not apply to the differences between normal and dystrophic muscle. As shown in Fig. 8, the nucleic-acid content of muscles containing small amounts of creatine was increased. This is in close agreement with the histological evidence which showed an intense nuclear proliferation. A similar increase in the amount of lipid phosphorus was found. When the lipid phosphorus was expressed as mg/g total lipid, the data were too variable to warrant a conclusion that the concentration of phospholipin in the fat phase had undergone any change in the dystrophic muscle.

Composition of the liver and brain

The analytical results obtained on the brains of the sixteen calves are summarized in Table 16.

The smaller dry-matter content of the brains of the calves given cod-liver oil or deprived of vitamin E was significant ($P=0·05$). This suggests that the increase in the weight of the brain in proportion to body-weight in dystrophic animals previously reported (Blaxter *et al.* 1952) was due to water. There was not an exact correlation, however, between the water content of the brain and the water content of muscle. Thus the brain of calf no. AO.E. 1 contained 19·5 % dry matter and that of calf no. AO.O. 1 contained 19·3 %. Their skeletal musculature, however, contained 21·2 and

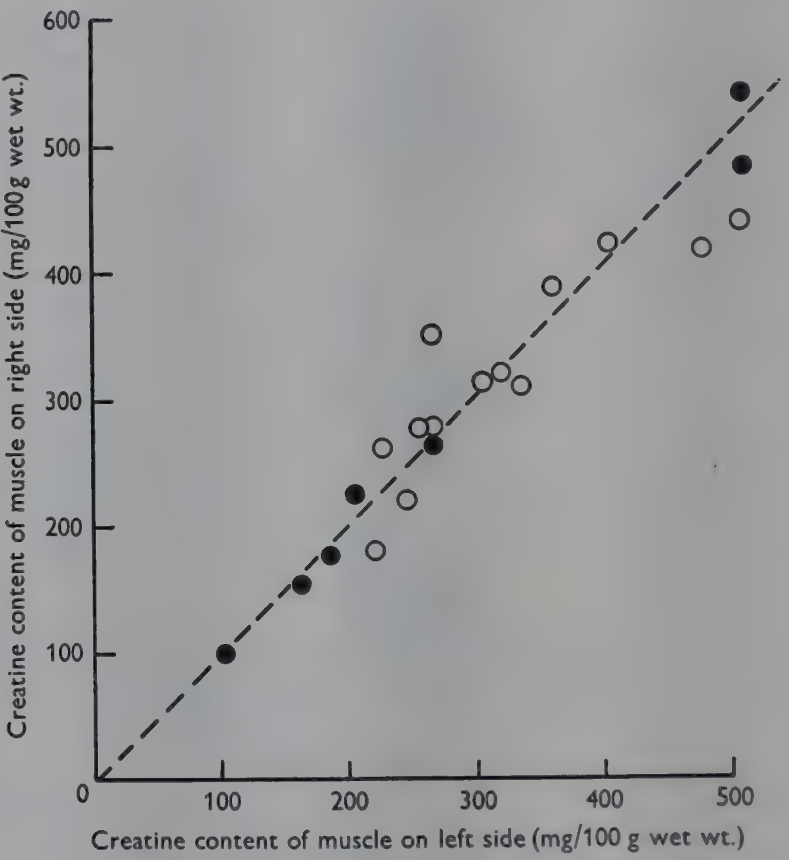

Fig. 6. Bilateral symmetry of the creatine content of the muscles. ●, anterior limb muscles of calf no. CLO.O. 3; ○, anterior and posterior limb muscles of calf no. CLO.E. 3.

Table 15. *Percentage distribution of total phosphorus in skeletal muscles of sixteen calves*

	Vitamins A and D supplied by			
	Cod-liver oil		Solution in arachis oil	
Phosphorus fraction	Without vitamin E	With vitamin E	Without vitamin E	With vitamin E
Acid-soluble + inorganic phosphorus	66·1	64·4	72·4	74·3
Lipid phosphorus	22·1	22·3	15·3	17·1
Nucleic-acid phosphorus	11·8	12·6	12·3	8·6
Total phosphorus (mg/100 g muscle)	1103	1075	949	926

18·8 % dry matter, respectively. The total lipid content of the brain was not affected by treatment. It would appear, however, that the cholesterol content of the brain was less in the dystrophic animals. Statistical analysis showed that this effect was not significant. The mean composition of the livers of the calves is given in Table 17.

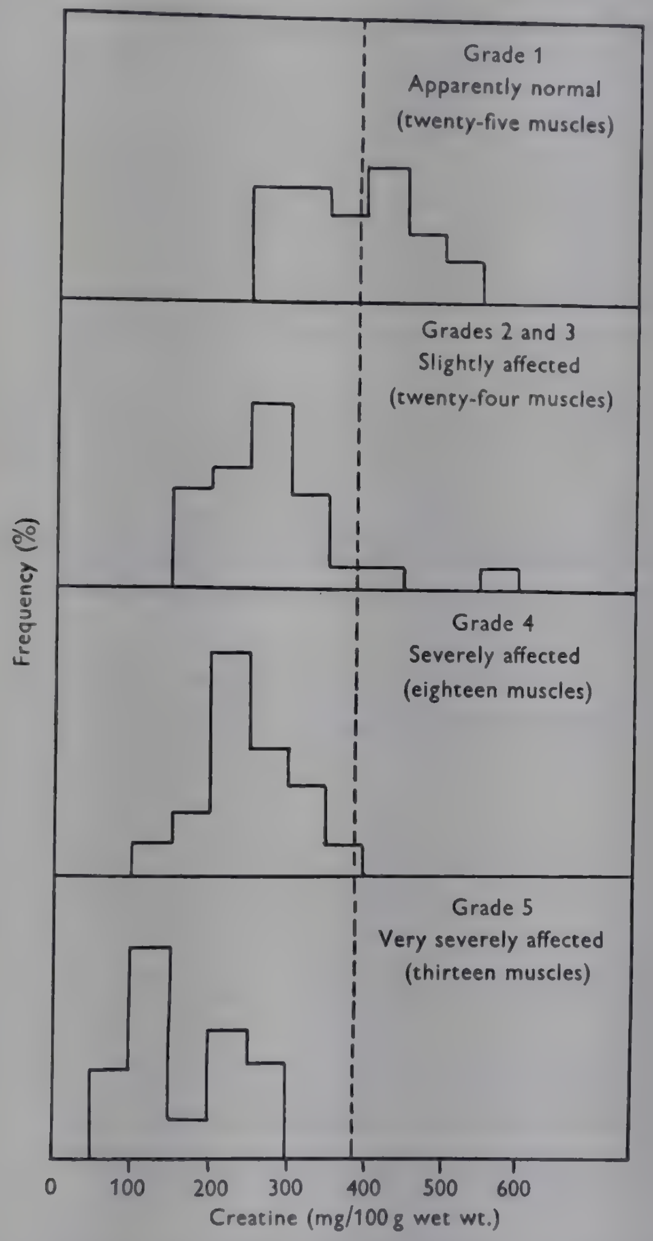

Fig. 7. Percentage frequency-distributions of the creatine content of muscles from dystrophic calves, classified according to the dystrophic involvement of the muscle. The dotted line represents the mean muscle-creatine content of normal calves. The frequency distribution at the top of the diagram may be compared with the distribution of muscle creatine of normal calves, given in Fig. 5.

The livers of those calves given α-tocopherol contained more dry matter than those of calves deprived of vitamin E. The livers of the calves given vitamins A and D in solution in arachis oil contained considerably more fat than those that received cod-liver oil. There was no significant difference due to vitamin E administration. There was no large change in the cholesterol or lipid-phosphorus content of the liver suggesting that the increase in fat content was largely the result of deposition in the liver

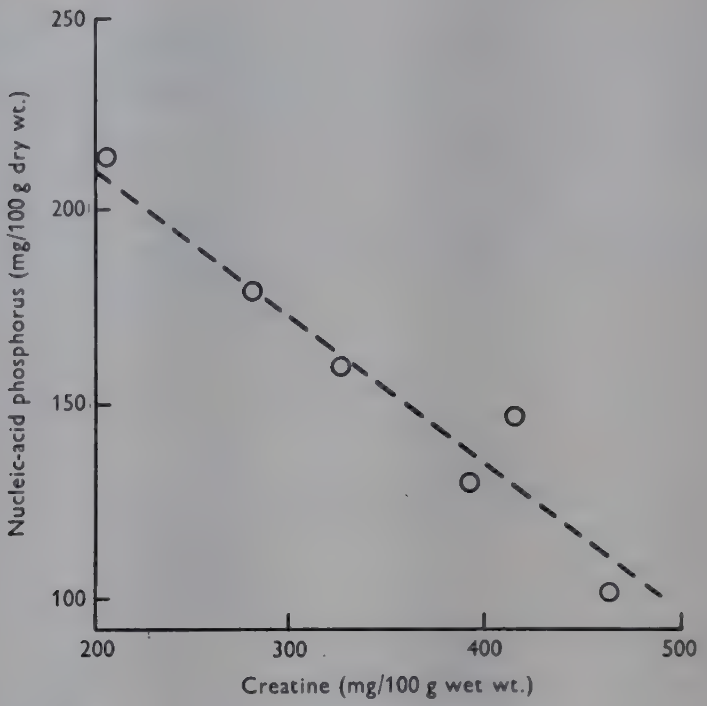

Fig. 8. Relation between the mean creatine content and the mean nucleic-acid content of muscles. Each point represents the mean result of either four or five simultaneous determinations of nucleic-acid, phosphorus and creatine content of skeletal muscles. Muscles from all sixteen calves are included.

Table 16. *Mean dry-matter content and mean content expressed on the dry basis of total lipid, cholesterol, lipid and total phosphorus of the brains of the sixteen calves, four in each group*

Constituent	Vitamins A and D supplied as			
	Cod-liver oil		Solution in arachis oil	
	Without vitamin E	With vitamin E	Without vitamin E	With vitamin E
Dry matter (%)	18·41	18·46	18·74	19·29
Total lipid (%)	43·5	41·5	43·4	42·7
Cholesterol (%)	8·4	8·7	9·4	10·7
Lipid phosphorus (mg/100 g)	627	447	499	510
Total phosphorus (%)	1·6	1·4	1·5	1·5
Cholesterol as percentage of the total lipid	19·3	20·9	21·6	25·0
Lipid phosphorus (mg/100 g total lipid)	14·4	10·8	11·5	12·0

Table 17. *Mean dry-matter content and mean content expressed on the dry basis of total lipid, cholesterol, lipid and total phosphorus of the livers of the sixteen calves, four in each group*

	Vitamins A and D supplied by			
	Cod-liver oil		Solution in arachis oil	
	Without vitamin E	With vitamin E	Without vitamin E	With vitamin E
Dry matter (%)	24·19	24·50	23·97	25·64
Total lipid (%)	3·85	5·35	9·82	7·64
Cholesterol (%)	0·92	0·94	1·15	1·04
Lipid phosphorus (mg/100 g total lipid)	378	371	363	319
Total phosphorus (%)	1·28	1·22	1·22	1·06

of neutral fat. Such deposition did not appear in any way related to the incidence of dystrophy and probably reflected the greater terminal failure of appetite in the calves given cod-liver oil.

DISCUSSION

The results of analyses of the muscles support the conclusions reached in the previous paper (Blaxter *et al.* 1952) that a dietary deficiency of α-tocopherol results in muscular dystrophy; that the inclusion of cod-liver oil leads to an exacerbation of the disease and that only 15–18 ml. cod-liver oil is sufficient to render ineffective the protective action of a daily supplement of 50 mg DL-α-tocopheryl acetate. These conclusions, however, were only statistically valid where the means of a large number of analyses for each calf were examined. Where only one or two muscles from each calf were analysed, statistical treatment of the resultant means often failed to show significance at the 5 % level. This is but a reflexion of the considerable variation in intensity and incidence of dystrophic lesions in the muscles of the affected individual. Analyses for constituents other than creatine, dry matter and ash were generally made on the biceps femoris. In several calves this muscle was normal in appearance, and, as the creatine analyses subsequently showed, not necessarily representative of the carcass as a whole. The results, however, may be used to demonstrate differences between normal and dystrophic musculature by methods involving cross classification, and it is with these differences, irrespective of treatment group, that this discussion is concerned.

Comparison of our results for dystrophic calf muscle with published analyses of dystrophic muscles from other animal species has already been given. The results with the calf demonstrate that the contents of ash, sodium, calcium, fat, cholesterol, nucleic acid, stroma protein, collagen and lipid phosphorus of dystrophic muscle were higher than in the normal, whereas the dry-matter, potassium, total nitrogen, globulin nitrogen, iron and creatine contents were lower in affected muscle. Such data are difficult to interpret, for they are expressed as percentages, and thus a change in the concentration of only one component must necessarily involve a change in the concentration of the others. The increase in the percentage of cholesterol, for instance, could be due either to a gross abnormality in cholesterol metabolism or to a large decrease in the amount of some other component of the muscle. The possibility exists that both interpretations may be necessary to account for the total increase. Some elucidation is made possible by a consideration of relative changes in the composition of the muscle. With the observed increase in cholesterol it was shown that the ratio of free to esterified cholesterol, and the percentage of cholesterol in the total lipid of the muscle, were unaffected in dystrophy. The lipid phosphorus expressed as a percentage of the total lipid was similarly unaffected. Together, these results suggest that increases in the concentration of cholesterol, cholesteryl ester, lipid phosphorus and total lipid in dystrophic muscle were secondary to a major change in some other fraction. By analogous reasoning the increase in water content, which was linearly related to the increase in ash content, may be ascribed to a change in the proportion of extracellular to intracellular water. This would account for the marked increase in sodium content and the fall in potassium content. The nitrogenous fraction is more difficult to interpret. Expressed as a percentage of the total nitrogen, the content of stroma proteins, collagen and nucleic acid increased, whereas the globulins, creatine and muscle haemoglobin (measured through its iron content) decreased. In the previous paper (Blaxter *et al.* 1952) it was shown that animals deficient in vitamin E stored less body nitrogen. This suggests that the primary change in the nitrogenous constituents was a decrease in the amount of muscle globulins synthesized, and that the observed changes in concentration of stroma proteins, collagen and nucleic acid were largely the result of this fall. The conclusion that the major biochemical defect in dystrophic muscle is a decline in globulin synthesis or, more probably, an increase in globulin breakdown, has much to support it. Firstly, in one of our earlier cases a myoglobinuria was observed, and in the naturally occurring disease of *weisses Fleisch* (Hjärre & Lilleengen, 1936) myoglobinuria is common. Secondly, a reduction of the globulin content of the cell would result in considerable changes in osmotic equilibrium and would account for the observed changes in the electrolyte content of dystrophic muscles. Thirdly, the observed decrease in the globulin content would be sufficient to account for a large part of the increase in the total lipid content and lipid fractions. Lastly, the histological appearance of the muscle, indicating a marked loss of contractile elements followed by a proliferation of sarcolemmal tissue and slight leucocytic infiltration, is in complete agreement with the contention that the globulins of the muscle are lost in large amounts (Macdonald, Blaxter, Watts & Wood, 1952). Secondary changes undoubtedly account for the increase in the calcium content of the muscle and for at least part of the increase in nucleic-acid content.

One characteristic of dystrophic musculature in other species is the increase in its oxygen uptake in vitro. Phosphorylating enzymes have been shown to decrease in concentration, while succinic dehydrogenase shows a remarkable increase. In view of the above discussion the concentration of these enzymes may reflect the change in gross composition of the tissue rather than biochemical abnormalities of the muscle cell. In this respect it is of interest that succinic dehydrogenases are closely associated with the nucleic-acid content of the tissue, whereas phosphorylating enzymes are in general cytoplasmic.

SUMMARY

1. Data relating to the chemical composition of muscles of the calves described in a previous paper (Blaxter *et al.* 1952) are given.

2. The dry-matter content of dystrophic muscles was lower than that of normal muscles.

3. The ash content of dystrophic muscles was greatly increased. Those muscles that were the most seriously affected contained nearly three times as much ash as normal muscles.

4. There was a considerable calcification of the affected muscles, an increase in their sodium content and a decrease in their potassium content. The phosphorus content was slightly increased but, on the basis of only a few analyses, magnesium content remained within the normal range.

5. The total lipid content of the dry muscle increased in dystrophic muscle. The total and free cholesterol and phospholipin content of the dry muscle also increased.

The percentage of cholesterol and phospholipin in the total lipid of dystrophic muscle, however, was unchanged.

6. The total nitrogen content of dry dystrophic muscle was less than that of normal muscle. There was an increase in the amount of collagen nitrogen and in the stroma-protein nitrogen. The total globulin nitrogen was markedly decreased, the nucleic-acid content increased and the iron content of the muscle decreased.
7. In dystrophic muscles the creatine content expressed either on a wet or a dry basis was greatly reduced. The reduction in the creatine content of the skeletal muscles was bilaterally symmetrical in the individual calf.
8. The classification of affected muscles by visual examination agreed only partly with their creatine content.
9. Statistical analysis of the analytical results confirmed the conclusions on the effect of presence of cod-liver oil and absence of vitamin E in the production of muscular dystrophy.
10. Data relating to liver and brain composition are presented.
11. The results are discussed, and it is pointed out that the results can be explained by a major change in the synthesis or destruction, or both, of globulins in dystrophic musculature. An increase in fat, cholesterol, lipid phosphorus, stroma protein and collagen as well as oedema of the muscle could be accounted for in this way. Secondary changes account for the calcification and part of the increase in nucleic-acid content.

Our thanks are due to Mr J. A. F. Rook for the determinations of the sodium and potassium content of the muscles. We are grateful to Miss G. Breckenridge and Miss M. Cunningham for their technical assistance.

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The Nutrition of the Young Ayrshire Calf

10. Histopathology of Muscular Dystrophy and its Relation to Muscle Chemistry

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(*Received* 27 *March* 1951)

The purpose of this paper is to describe the histological appearance of tissues from the dystrophic calves reported by Blaxter, Watts & Wood (1952), to compare the dystrophic lesions with those found in naturally occurring muscular dystrophy of cattle (Hjärre & Lilleengen, 1936*a*, *b*, Vawter & Records, 1947; Slagsvold, 1925), and to relate the chemical composition of the muscle to its histological appearance.

METHODS

As described in a previous paper (Blaxter *et al.* 1952), four experimental groups of calves were involved; two receiving cod-liver oil as a source of vitamin A and D activity and two a solution of pure vitamins A and D in arachis oil. Vitamin E was given to animals in one of each of these groups, the other group receiving none. The calves are referred to by treatment and replication number as previously described. Blocks of muscle were fixed with 5 % formaldehyde or 5 % formol saline. Sections were stained by Mayer's haemalum and 5 % aqueous eosin. Gallego's method (see Langeron, 1949) was used to demonstrate connective tissue. Sections were also stained with phosphotungstic acid and haematoxylin.

RESULTS

Table 1 summarizes the results of the examination of skeletal and cardiac musculature, together with details of the macroscopic appearance of the muscle and its chemical composition. Both normal muscles from normal animals and 'normal' muscles from dystrophic animals were included in this study.

The agreement of the results was on the whole good. Muscle classified as normal macroscopically and containing the usual quantities of dry matter, total nitrogen and creatine was found to be normal when examined histologically. There were, however, several discrepancies. The flabby heart of calf no. CLO.E. 1, although containing subnormal amounts of creatine, was histologically normal, and the semimembranosus of calf no. CLO.E. 4, though of normal composition and appearance, nevertheless showed an increased number of nuclei. These discrepancies are partly due to the sampling errors involved when a small piece of muscle is excised for histological study and the remainder of the muscle macerated for chemical analysis, as well as to the subjective judgement involved in the classification of muscles into groups on the basis of their macroscopic appearance. It may be noted from the table that the severe lesion of the biceps femoris of calf no. CLO.O. 3 was bilaterally symmetrical. It may also be seen that where the lesion was localized and almost circumscribed, as in the heart of calf no. AO.O. 4, the surrounding tissue had a normal appearance.

On the basis of the experimental material presented in Table 1 a tentative account of the histogenesis of the lesion may be inferred. The earliest change appeared to be a swelling of the muscle fibre and a loss of staining power of the muscle bundles or small groups of fibres (Pl. 1, 1–3). In haematoxylin- and eosin-stained sections a deepening of the staining as the result of a closer arrangement of the anisotropic discs of the muscle fibres was found (Pl. 1, 5). The most significant change, however, was the reaction of the sarcolemmal nuclei (Pl. 1, 4, 5). These swelled in the early stages and then multiplied roughly in inverse proportion to the atrophy of the muscle cells, thus apparently maintaining the total amount of tissue (Pl. 1, 6). There was no gross fibrosis or fatty infiltration (Pl. 2, 2). The advanced lesion was remarkable for the enormous proliferation of the sarcolemma and the almost complete absence of muscle (Pl. 1, 6; Pl. 2, 1). The muscle remaining was fragmented, but retained its staining properties and its striation, though, functionally, it was clearly of no value (see Pl. 1, 6 and Pl. 2, 1). The blood vessels were normal and the nervous tissue that was present was also normal. Rarely, polymorphonuclear leucocytes were seen phagocytosing a necrotic bundle, but their rareness indicated that, essentially, the lesion was degenerative and not inflammatory.

The changes in the cardiac musculature evidently followed the same general sequence and the terminal phase is shown in Pl. 2, 1.

DISCUSSION

The lesions that we have observed in calves fed on vitamin E-deficient diets agree well with the lesions found in other species. There are, however, three discrepancies. Firstly, in the calf the muscle cells retained their striated appearance despite their considerable fragmentation, a conclusion supported by Vawter & Records (1947) on the basis of their examination of naturally occurring cases of *weisses Fleisch*. With dystrophic rats, Pappenheimer (1948) has stated that one of the earliest changes to be found was a loss of striation and in pseudo-hypertrophic dystrophy in man the striations also disappeared in the early stages of the disease (Bicknell & Prescott, 1946). Hjärre & Lilleengen (1936*a*, *b*) refer to a ‘diskoider Zerfall’. This could be interpreted as a loss of striation, but such a loss is not apparent in the photographs which they have published.

The second difference between the micropathological appearance of the lesion in our calves and those in other species concerns the sarcolemmal proliferation and negligible infiltration by polymorphonuclear leucocytes. The naturally occurring cases of *weisses Fleisch* that have been described are characterized by marked variation in the interpretation of the origin of the masses of proliferating nuclei. With the exception of Nieberle (1926) all workers concur that no reactively inflammatory

Table 1. *Description of gross lesions, histopathology and chemical composition of normal and dystrophic muscles in calves*

Calf no.	Muscle	Composition of muscle			Gross appearance of muscle	Histological appearance of muscle
		Dry matter (%)	Nitrogen (%)	Creatine (mg/100 g)		
CL.O.O. 3	Heart	22·9	2·64	227	Normal	Normal
AO.E. 4	Heart	20·6	2·79	320	Normal, dark in colour	Normal
AO.E. 1	Heart	20·0	2·38	229	Normal	Normal
AO.E. 2	Heart	20·0	2·54	235	Normal	Normal
CL.O.E. 1	Heart	20·2	2·60	161	Normal, but very flabby	Apparently normal
CL.O.O. 1	Heart	19·9	2·84	246	Normal	Normal
CL.O.E. 2	Heart	19·9	2·68	177	Pale, otherwise normal	Scattered bundles of nuclei throughout section examined, probably representing an early stage of dystrophy
CL.O.O. 4	Heart	19·5	2·51	276	Small haemorrhages in the auriculo-ventricular valves but no indication of dystrophy	Normal
AO.O. 4	Heart	20·6	2·73	235	Two areas of necrosis on outer wall of left ventricle and one large internal dystrophic lesion with smaller other lesions	(*a*) Apparently unaffected area of muscle. Normal (*b*) Severely dystrophic area. Large tracts of muscle replaced by proliferated sarcolemmal cells, each in a pocket of delicate connective tissue. Fine bands of connective tissue but no coarse replacement-fibrosis apparent. Slender wisps of muscle remained unaffected. No leucocytic infiltration
AO.E. 4	Rectus femoris	22·1	3·36	463	Normal	Some slight variation in staining power of muscle cells but normal in other respects
	Left long head of triceps	21·9	3·28	468	Normal	Normal
	Right long head of triceps	21·8	3·28	412	Normal	Normal
CL.O.E. 4	Biceps femoris	21·6	3·19	418	Normal	Normal
	Semi-membranosus	21·8	3·26	512	Normal	Very slight increase in the number of nuclei present

AO.O. 3	Rectus femoris	20·6	2·91	322	Two large white areas in centre of muscle—severely affected	Several areas of increased pigmentation of muscle bundles with possibly local increases in the number of sarcolemmal nuclei. No fibrosis, and none of the muscle atrophic
AO.O. 4	Rectus femoris	22·3	3·20	417	One large pale area present	A diffuse increase in the number of sarcolemmal nuclei. Majority of muscle bundles hyalinized with loss of striations; some swollen
	Long head of triceps	20·4	2·74	261	One very large white area	A severe lesion with marked atrophy. Loss of striation and staining power throughout the whole section. Intense sarcolemmal proliferation but no evidence of leucocytic proliferation or of an increase in connective tissue
CLO.O. 3	Left biceps femoris	18·0	2·36	138	Whole muscle dead white in colour with small streaks apparently normal	A very severe lesion in which the proliferated sarcolemma was completely dominant. Normally staining muscle present only in flecks. Muscle clearly non-functional. Several of the muscles hyalinized. Multinucleate giant cells had formed from the sarcolemmal tissue. No leucocytes seen
	Right biceps femoris	—	—	—	Whole muscle dead white and the extent of dystrophy indistinguishable from that of the left biceps femoris	Lesion almost identical with that of the left biceps femoris
	Long head of triceps	18·1	2·42	205	Centre of muscle entirely white, fading to a more normal colour at the periphery	Lesion comparable to that found in the left biceps femoris
CLO.O. 4	Vastus medialis	17·8	—	208	Whole muscle white in colour	Very severe lesion comparable to that of left biceps femoris of calf CLO.O. 3
	Long head of triceps	16·8	2·21	200	Large white non-circumscribed areas throughout the muscle	Very severe lesion comparable in every respect to that of left biceps femoris of calf CLO.O. 3

changes take place in the interstitial tissues, and that only in exceptional cases are histiocytes and leucocytes observed (Ziegler, 1926; Vawter & Records, 1947; Hjärre & Lilleengen, 1936*a*, *b*). The nuclear masses have been regarded by Hjärre & Lilleengen as regenerating muscle nuclei beneath the sarcolemma, and Vawter & Records (1947) interpret them as a 'dense mass of muscle nuclei, polymorphonuclear leucocytes, histiocytes and giant cells'. Our findings are not so. Jones & Read (1948), reporting on a naturally occurring case of muscular dystrophy in a foal, emphasize that the nuclear masses are of sarcolemmal origin, but in their animal, in which the immediate cause of death was broncho-pneumonia, lymphocytes and neutrophils were also present. In the suckling rat, Olcott (1938) has shown that marked sarcolemmal proliferation occurs, and this too has been the conclusion of Follis (1948). Pappenheimer (1948), however, states that in the rat there is a 'strong inflammatory response' to the initial segmentation and hyaline degeneration of the muscle cell. In the rabbit, the multinucleate masses are thought to be fused polymorphonucleate leucocytes and histiocytes (Pappenheimer, 1940–1). It would appear, therefore, that part of the differences may perhaps be due to the difficulties of interpretation of sections in which such marked changes have occurred. From the present studies with calves, however, and published findings with rabbits, guinea-pigs and men, it appears that irrespective of species an attempt is made to maintain constant the volume of the dystrophic muscle. With the calf this is done by massive sarcolemmal proliferation, with other species by excessive fat deposition. This may reflect the quite normal differences in anabolic processes between young calves, which store negligible fat but very large amounts of protein, and mature rabbits, guinea-pigs and men, in which protein deposition is normally minimal and fat deposition predominates.

The third point of difference concerns the incidence of calcification. This aspect has already been referred to when discussing the biochemical results (Blaxter & Wood, 1952). In naturally occurring cases, Vawter & Records have commented on the extensive calcification of the muscles, and Hjärre & Lilleengen (1936*a*, *b*) have stated: '...mitunter liegen feine Ca-Körner reihenweise zwischen den Myofibrillen, was eine abnorme deutliche Längsstreifung veranlasst...'. We have not however observed such longitudinal striation in our sections.

From these results and comparisons, additional support is thus given to the contention that the naturally occurring disease of *weisses Fleisch* in calves is very closely comparable to the vitamin E-deficiency state which we have produced.

SUMMARY

1. A description of the histology of muscular lesions observed in calves is presented.

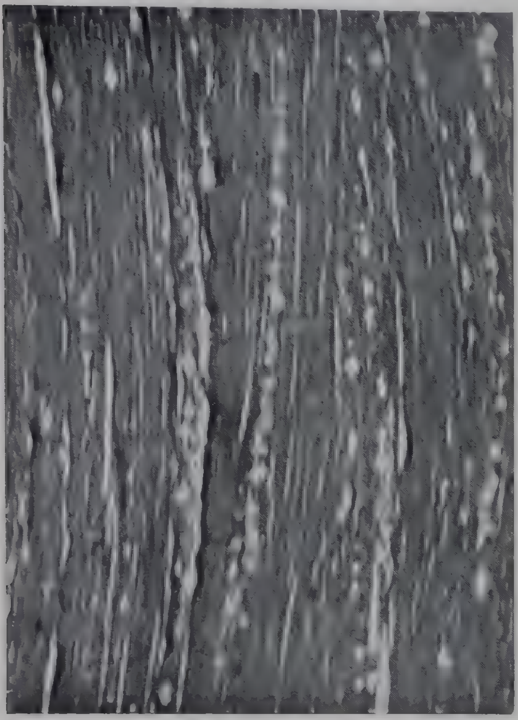
2. It was shown that the macroscopic, biochemical and histological data were in good agreement.

3. Differences between the histology of the lesion in the dystrophic calf and in other dystrophic animals are emphasized.

We are obliged to Mr W. Mason and Mr Evett of the Children's Hospital, Glasgow, for the photographs.

A. M. MACDONALD, K. L. BLAXTER, P. S. WATTS AND W. A. WOOD. *Plate* 1

NUTRITION OF THE AYRSHIRE CALF. 10



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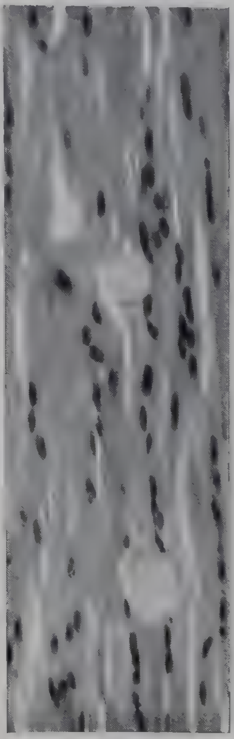

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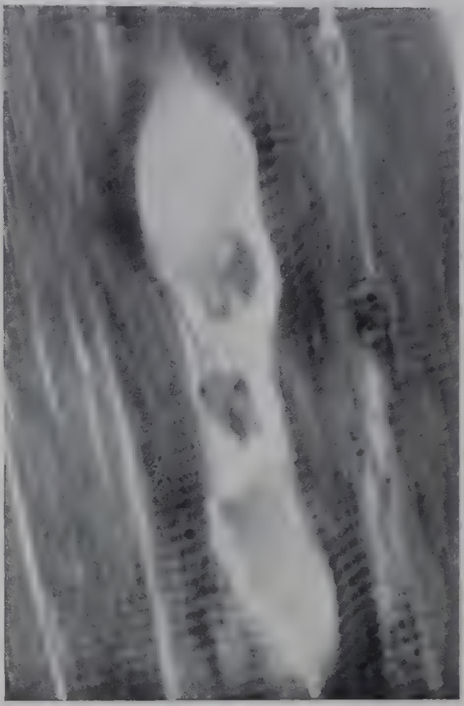

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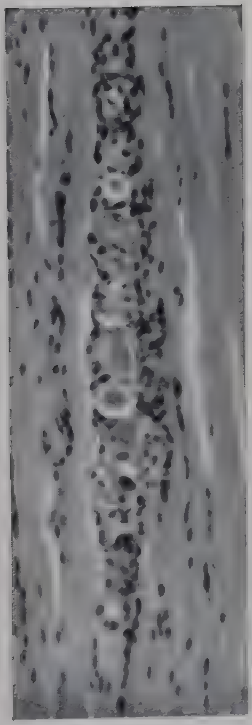

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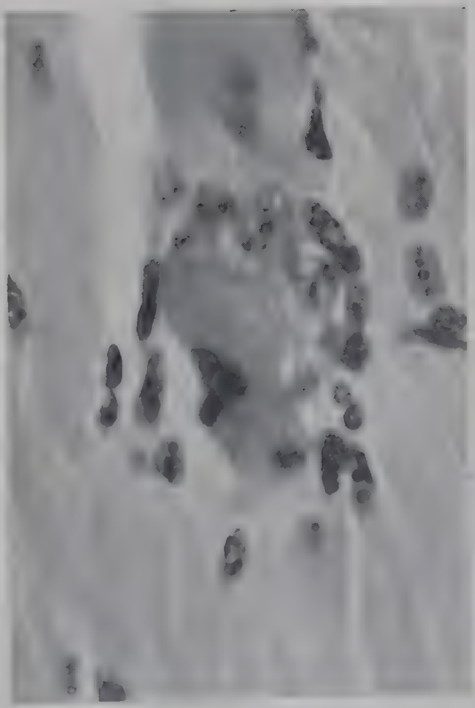

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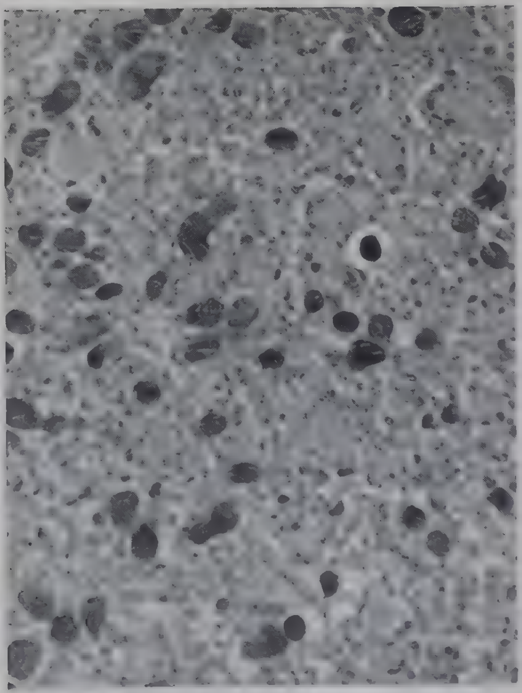

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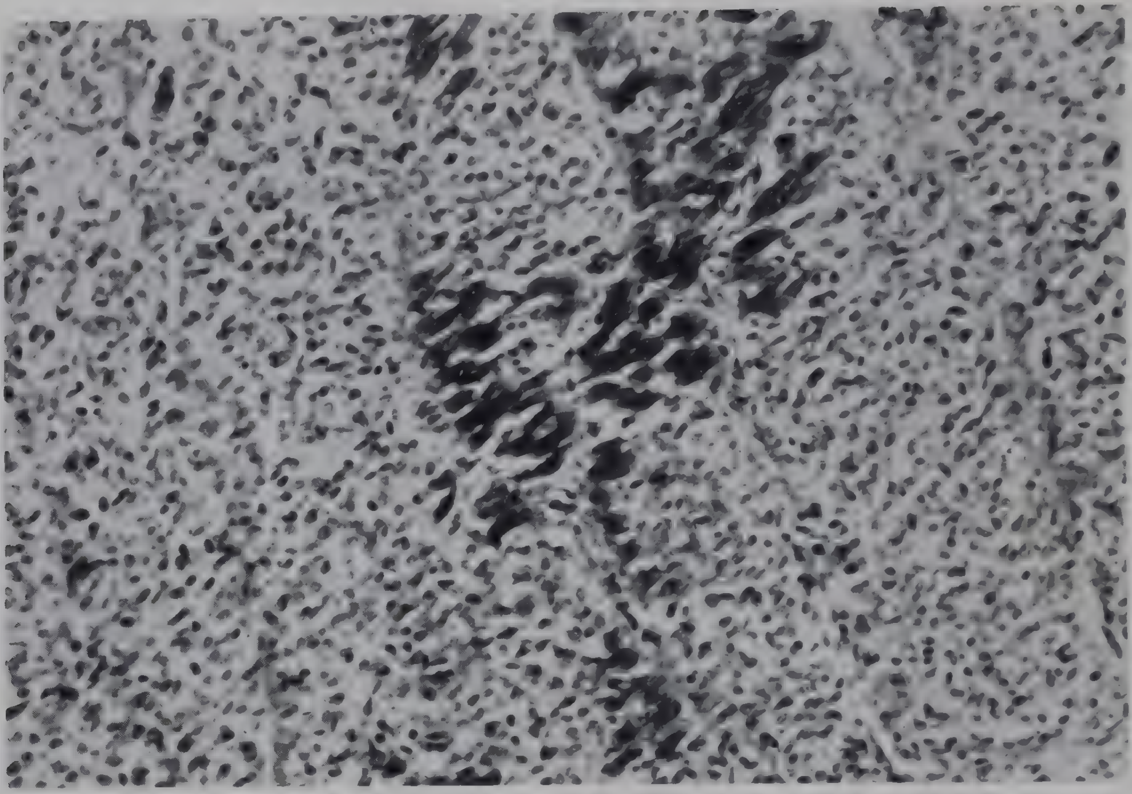

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EXPLANATION OF PLATES

PLATE 1

1. Low-power magnification of skeletal muscle of calves showing affected fibres appearing as white beaded streaks in the midst of the dark normally staining muscle. The lesion is punctate and clearly defined. Phosphotungstic-acid haematoxylin stain. × 30.

2. Very early lesion of skeletal muscle of calves. Small hyaline nodes are shown in a muscle bundle or possibly fibre, surrounded by normal muscle and a normal density of sarcolemmal cells. Mayer's haemalum and eosin. × 100.

3. Lesion of skeletal muscle of calves similar to that in Pl. 1, 2. Note the isolated hyalinization surrounded by normally striated muscle. Phosphotungstic-acid haematoxylin stain. × 370.

4. Beginning of sarcolemmal proliferation around an affected muscle bundle in skeletal muscle of calves. Mayer's haemalum and eosin. × 70.

5. High-power magnification of the early sarcolemmal proliferation in skeletal muscle of calves. Irregular striation of the affected fibres is visible and the initial hyaline lesion is now necrotic. Mayer's haemalum and eosin. × 370.

6. Advanced lesion in a cross-section of skeletal muscle of calves. Great sarcolemmal proliferation; some apparently normal dark-staining muscle remains, but several muscle bundles may be seen which have completely lost their staining reactions. Phosphotungstic-acid haematoxylin stain. × 70.

PLATE 2

1. Dystrophic heart muscle of calf no. AO.O. 4. A wisp of normally staining muscle remains in a massive sarcolemmal proliferation. Phosphotungstic-acid haematoxylin stain. × 70.

2. Cross-section of severely dystrophic muscle of calves stained by the Gordon-Sweets method for reticulum. × 100.

The Assay of Vitamin B_{12}

7. Effect of Dietary Lactose and of the State of Maternal Nutrition on the Growth Response of the Rat to Vitamin B_{12}*

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(*Received* 8 *June* 1951)

Vitamin B_{12} improves the growth of rats maintained on diets of vegetable origin (Emerson, Wurtz & Zanetti, 1949). The effect is greater in animals from parents fed on such diets based on soya with adequate added minerals and vitamins (Emerson *et al.* 1949; Dryden, Hartman & Cary, 1949; Bosshardt, Paul, O'Doherty, Huff & Barnes, 1949). Addition of lactose to the diet depresses the growth of rats (Ershoff, 1949); the effect may be overcome by the administration of vitamin B_{12} (Hartman, Dryden & Cary, 1949).

The work described here was carried out during development of a procedure for assaying vitamin B_{12} on rats, and is concerned with the effects of dietary lactose and of the nutrition of the mother on the response of young rats to vitamin B_{12}.

EXPERIMENTAL

Animals. Albino rats of the WAG strain (Bacharach, Cuthbertson & Thornton, 1949) were used throughout. In tests to determine the growth-promoting effect of vitamin B_{12} equal numbers of weanling male and female animals, weighing about 30 g, were used. The mothers of the experimental animals were from the breeding colony and had been maintained on stock diet RBSS 10*a* (Table 1) with weekly supplements of cabbage and 3 g liver, or had been placed on the soya-glucose deficient diet RB 12 S/2 (Table 1) before mating. The effect of vitamin B_{12} supplements on growth rate was studied in normal stock weanlings and in first, second and third litters from does maintained on the soya-glucose diet.

Maintenance of animals. The mothers were housed in wooden breeding cages with wood-wool litter; food and water were unrestricted.

The young animals to be used in the growth experiments were weaned at 21 days, and were subsequently maintained in wire cages on screen bottoms, two animals in each cage, for 28 days. During this period their supply of water and experimental diet was unrestricted. The diets used were one or other of the soya-diets RB 12 S/2 and RB 12 S/4 (Table 1).

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* Parts 1–6 have been published as follows:
Part 1. Cuthbertson, W. F. J. & Lloyd, J. T. (1951). *J. gen. Microbiol.* **5**, 416.
Part 2. Lees, K. A. & Tootill, J. P. R. (1951). *Biochem. J.* **50**, 455.
Part 3. Cuthbertson, W. F. J., Pegler, H. F. & Lloyd, J. T. (1951). *Analyst*, **76**, 133.
Part 4. Emery, W. B., Lees, K. A. & Tootill, J. P. R. (1951). *Analyst*, **76**, 141.
Part 5. Cuthbertson, W. F. J., Pegler, H. F., Quadling, C. & Herbert V. (1951). *Analyst*, **76**, 540.
Part 6. Harrison, E., Lees, K. A. & Wood, F. (1951). *Analyst*, **76**, 696.

Procedure with experimental animals fed on the soya-glucose diet RB12S/2. Weanling rats from mothers on normal stock diet were used and also animals from the first, second and third litters of mothers maintained on the soya-glucose diet RB12S/2. The animals were divided into groups, each containing equal numbers of males and females, and were dosed with vitamin B_{12} by stomach tube twice a week. Observations were made for 28 days.

Table 1. *Percentage composition of the diets*

Stock diet RBSS 10*a*

Wholemeal wheat flour	75
Full-cream, roller-dried milk	20
Dried yeast	5

Supplement of liver, 3 g, and green leaves, 3 g/week

Soya diets ...	RB12S/2	RB12S/4
Full-fat soya flour	70	72
Glucose	24	—
Lactose	—	22
Salts (Hubbell, Mendel & Wakeman, 1937)	4	4
SKADE 3*	2	2
Vitamin supplement†		

* SKADE 3 (fat-soluble vitamin solutions):

Vitamin A concentrate	2×10^5 i.u.
Calciferol	0·25 mg (10^5 i.u.)
α-Tocopheryl acetate	14·2 g
Menaphthone	100 mg
Arachis oil to	1000 g

† Vitamin supplement/kg diet (mg):

Inositol	220	Aneurin HCl	30
Nicotinic acid	100	Riboflavin	30
Ca-D-pantothenate	100	Pyridoxin HCl	8
p-Aminobenzoic acid	75	Folic acid	1

Biotin 0·2

Procedure with experimental animals fed on the soya-lactose diet RB12S/4. In these experiments the weanlings were obtained from the stock colony or from mothers maintained on the soya-glucose diet. The weanlings were themselves maintained on the soya-lactose diet, but were otherwise treated in the same way as the animals that in the preceding experiment had been fed on the glucose diet.

RESULTS AND DISCUSSION

Soya-glucose diet. The growth increments found are summarized in Table 2, which shows the growth also of normal animals on stock diet under comparable conditions. The figures show that normal weanling rats on the soya-glucose diet grew at a fair rate even in the absence of vitamin B_{12}, but that weanlings from mothers maintained on the soya-glucose diet grew poorly unless vitamin B_{12} was given. It can be seen also

Table 2. *Mean weight increases* (*g*) *of groups of weanling rats maintained for* 28 *days on a soya-glucose diet* (*RB* 12 *S*/2) *deficient in vitamin* B_{12}, *and given various supplements of vitamin* B_{12}

(Vitamin B_{12} was administered twice weekly by stomach tube)

Exp. no.	Origin of animals	No. of animals	Maternal diet	Supplement of vitamin B_{12} (μg/day) 0	0·04	0·08	0·16	0·32	0·64	1·0	2·5
1	Stock colony	8	Stock RBSS 10*a*	58·5	—	—	—	—	—	69·1	72·0
2	First litter	12	Soya-glucose RB 12 S/2	46·8	54·1	58·5	68·9	72·5	68·0	—	—
3*a*	Second litter	16	RB 12 S/2	48·5	50·3	53·4	50·3	59·0	—	—	—
3*b*	Second litter	8	RB 12 S/2	—	47·7	51·1	61·2	63·1	69·9	71·3	77·5
4	Third litter	6	RB 12 S/2	49·9	—	—	50·7	—	60·4	—	64·1
			Experimental animals fed on stock diet RBSS 10*a*								
5	Stock colony	8	RBSS 10*a*	—	—	—	—	—	—	72·0	—

After elimination of differences due to sex and treatment the standard deviation of an observation is ±10·5 g, i.e. the standard error of the mean weight increase of a group of eight animals is ±3·7 g.

Table 3. *Mean weight increases* (*g*) *of groups of eight weanling rats maintained for* 28 *days on a soya-lactose diet* (*RB* 12 *S*/4) *deficient in vitamin* B_{12} *and given various supplements of vitamin* B_{12}

(Vitamin B_{12} was administered twice weekly by stomach tube. Numbers in parentheses indicate the number of animals that died in each group)

Exp. no.	Origin of animals	Maternal diet	Supplement of vitamin B_{12} (μg/day) 0	0·04	0·16	0·64	2·56	5·0	7·5
6	Stock colony	Stock RBSS 10*a*	45·8 (1)	50·1	55·2	64·5	65·9 (1)	67·7	70·6
7	Stock colony	Stock RBSS 10*a*	36·9	—	—	—	—	—	—
8	First litter	Soya-glucose RB 12 S/2	25·1	32·2	37·0	46·0	48·2	54·2	—

After elimination of differences due to sex and treatment the standard deviation of an observation is ±9·2 g, i.e. the standard error of the mean weight increase of a group of eight animals is ±3·3 g.

that in the absence of vitamin B_{12} the growth rates of animals from the first, second and third litters of mothers on the soya-glucose diet were about the same. The responses of the animals given different doses of vitamin B_{12} in Exps. 1 and 2 show that a normal growth rate could be achieved by administering daily from 0·16 to 2·5 μg vitamin B_{12}. Consequently, it may be concluded that the rats on the soya-glucose diet were deficient only in vitamin B_{12}.

Comparison of the responses of first, second and third litters to vitamin B_{12} in Exps. 2, 3*a*, 3*b* and 4 shows that the amount of vitamin B_{12} required for growth was greater the longer the mothers had been maintained on the deficient diet. Thus, for a growth increment of about 60 g in 28 days, supplements of 0·08, 0·32 and 0·64 μg vitamin B_{12} a day were needed by animals from the first, second and third litters, respectively. Exps. 2, 3*a*, 3*b* and 4 show that the slope of the curves relating the amounts of vitamin B_{12} given to the growth increments became less with successive litters from mothers on the deficient diet. The fact that successive litters responded progressively less well to vitamin B_{12} might have been due to a decrease in the animals' ability to utilize vitamin B_{12}. This decrease may itself have been due to a continuous decrease in the reserves of some other factor needed for the utilization of vitamin B_{12}.

For assay purposes weanlings from the first litters of mothers on the soya-glucose diet would appear to be the most suitable, for supplements of vitamin B_{12} restored the growth rates to normal, whereas the growth obtained in absence of vitamin B_{12} was small. The response of such animals was satisfactory, for a daily supplement of 0·16 μg vitamin B_{12} induced during the observation period a growth response exceeding by about 20 g that of rats receiving no supplement.

Soya-lactose diet. The growth responses to graded supplements of vitamin B_{12} are shown in Table 3. Comparison of Tables 2 and 3 shows that the growth of normal weanlings on the soya-lactose diet was somewhat less than that of animals on the soya-glucose diet and born of mothers maintained on that diet (Table 2, Exp. 2). Normal growth could be restored by supplying adequate vitamin B_{12}, but the amount needed by animals on the soya-lactose diet was much greater than by those on the soya-glucose diet; thus 7·5 μg vitamin B_{12} a day were needed for maximum growth of stock animals on the soya-lactose diet, but only from 0·16 to 0·3 μg for that of animals on the soya-glucose diet even when their mothers had been maintained on the deficient diet.

With the soya-lactose diet the offspring showed even more markedly the effect of their mother's having received the soya-glucose diet, as can be seen from Exp. 8 (Table 3). The first litters from mothers fed on the soya-glucose diet grew only 25 g in 4 weeks on the soya-lactose diet, and supplements of up to 5 μg vitamin B_{12} a day did not allow them to grow normally.

From these results it would appear that normal stock animals given the soya-lactose diet could be used for vitamin B_{12} assay. The low growth caused by this diet could be completely remedied by adequate supplements of vitamin B_{12}, but the animals were less sensitive to vitamin B_{12} than those fed on the soya-glucose diet after their mothers had been maintained on the same diet.

The growth responses of the male rats were significantly higher, by about 20 %,

Table 4. *The effect of sex on the mean weight increases in g of groups* of rats fed on either diet RB 12 S/2 or RB 12 S/4 and dosed with varying amounts of vitamin B_{12} over a period of 28 days*

(Vitamin B_{12} was administered twice weekly by stomach tube)

Origin of animals	Parental diet	Experimental diet	Sex	Vitamin B_{12} (μg/day)									Standard deviation *s*	Regression in bracketed range *b*	Precision *b/s*
				0	0·04	0·08	0·16	0·32	0·64	2·56	5·0	7·5			
First litter	RB 12 S/2	RB 12 S/2	♂	51·7	[63·3	60·2	77·1]	75·6	73·5	—	—	—	±9·83 (*n′*=29)	+22·9	2·33
First litter	RB 12 S/2	RB 12 S/2	♀	43·3	[46·5	55·2	52·8	67·2	63·3]	—	—	—	±4·86 (*n′*=23)	+15·2	3·13
Stock colony	RBSS 10*a*	RB 12 S/4	♂	43·5	[56·0	—	61·2	—	74·5]	73·3	74·2	73·6	±7·30 (*n′*=20)	+15·4	2·11
Stock colony	RBSS 10*a*	RB 12 S/4	♀	51·3	[44·2	—	49·2	—	54·5]	60·2	62·2	65·8	±8·58 (*n′*=21)	+8·58	1·00

* Each group contained not more than seven and not less than three animals.

than those of the female animals in the same experimental conditions. The different responses of male and female rats fed either the soya-glucose or soya-lactose diet, and given various supplements of vitamin B_{12} are demonstrated in Table 4. From these results it is clear that on either diet the vitamin B_{12} requirement of the female rat is greater than that of the male. The statistical data presented in Table 4 show that male and female rats should be satisfactory for assay purposes if an appropriate range of vitamin B_{12} concentrations is employed.

SUMMARY

1. Weanling rats were maintained on a soya-glucose diet deficient in vitamin B_{12}. They required more vitamin B_{12} for growth when their mothers had been maintained on the same deficient diet than when the mothers had been maintained on a stock diet.

2. The offspring of does on the deficient diet gave graded growth responses to graded doses of vitamin B_{12} when maintained on the deficient diet.

3. The growth of such animals could be restored to normal by supplements of vitamin B_{12}.

4. The response to vitamin B_{12} decreased in successive litters from mothers on the deficient diet.

5. When the deficient diet contained lactose instead of glucose the growth rate was depressed, but the effect could be overcome by means of vitamin B_{12}.

6. Normal stock rats given the soya-lactose diet showed graded growth responses to graded doses of vitamin B_{12}.

7. When the offspring of mothers maintained on the soya-glucose diet were given the soya-lactose diet, their growth was adversely affected to a greater extent than that of the offspring of mothers maintained on a stock diet.

8. The vitamin B_{12} requirement of male rats was greater than that of females.

9. It is concluded that rats reared to weaning on stock diet could be used for vitamin B_{12} assay if the soya-lactose diet were given, but for this purpose it would be preferable to use animals from the first litters of mothers maintained on the soya-glucose diet and themselves maintained on this diet.

We wish to thank Mr Basil for mathematical assistance.

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The Administration of Cobalt by Different Routes to Lambs Maintained on a Low-Cobalt Diet

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(*Received 16 July 1951*)

The importance of cobalt in the nutrition of the sheep was discovered in Australasia (Lines, 1935; Marston, 1935; Underwood & Filmer, 1935; Askew & Dixon, 1936), and it was assumed that its action was exerted in the rumen, as neither horses nor rabbits maintained on cobalt-deficient land show signs of deficiency. Injection of cobalt salts or vitamin B_{12} does not prevent or cure deficiency (Marston & Lee, 1949), although some improvement may be noticed after the injection of large doses of cobalt (Ray, Weir, Pope, Bohstedt & Phillips, 1948; Keener, Percival, Ellis & Beeson, 1950).

There was no direct evidence to show how the rumen might be concerned until Tosic & Mitchell (1948) found that bacteria in the rumen concentrated cobalt from the surrounding media. One hypothesis suggested by this observation was that the rumen bacteria require cobalt for their normal metabolism. Concentration of trace elements by bacteria, however, may be fortuitous and the possibility also exists that, when the available cobalt is short, its concentration in bacterial cells may mean that none is available for essential purposes elsewhere. Masson (1950) has shown that some rumen micro-organisms disintegrate as they pass through the abomasum, and others as they pass along the small intestine; for this reason it is improbable that free cobalt is necessary in the lower parts of the alimentary tract. Injection of radioactive cobalt demonstrated the excretion of a part of it in the bile (Comar & Davis, 1947); in addition, traces of activity were found in both small and large intestines and even in the abomasum. This suggests that the action of cobalt may be confined to the rumen, but, as the counts recorded by Comar & Davis from the abomasum and intestines were very small, additional investigations were needed.

The experiments described here were designed to establish whether the action of cobalt could be localized to the rumen.

EXPERIMENTAL

General. Four experiments were carried out. The first was designed to test the adequacy of the ration with added or injected cobalt. The second was designed to ascertain whether nicotinic-acid deficiency was being caused by the excessive quantity of maize in the ration. The third and fourth were designed to study the effect of introducing cobalt into the alimentary tract caudally to the rumen and reticulum.

A modification of the ration used by Stewart (1947) was used to produce and maintain cobalt-deficient animals.

All lambs were kept individually in wooden pens treated with creosote; metal fittings were removed and the floors were slatted so that faeces dropped through. This is an important precaution, as Comar, Davis, Taylor, Huffman & Ely (1946) have shown that 65 % of radioactive cobalt introduced into the rumen is excreted in the faeces. Drinking water was placed in china bowls, and wooden feeding troughs were fitted with an overhanging wooden ridge to prevent the scatter of food. Kitchen cooking salt was always available in china bowls.

All nails or screwheads on the pens were painted over with a plastic paint to prevent the sheep licking them.

Source of animals

Experiment 1. The lambs used in the first experiment were obtained from a farm in the north of Morayshire; part of this land had been used as an aerodrome during the war and levelling operations had brought the subsoil to the surface.* Samples of soil taken from the part of the farm in question contained from 0·10 to 0·16 p.p.m. of cobalt soluble in 2·5 % acetic acid. In Scotland, 0·3 p.p.m. probably indicates an adequate cobalt concentration in normal soils.

Twenty-four Cheviot lambs, 60–80 days old, were placed on these pastures; twelve were maintained on untreated pasture and twelve others, to which were added a few late lambs, were maintained on an adjacent pasture that had been treated with cobalt sulphate at the rate of 2 lb./acre. When the two groups were weighed, approximately 2 months later, a marked difference had developed between them, as shown in Table 1.

Table 1. *Exp.* 1. *Weights of Cheviot lambs that had been kept for 2 months on treated and untreated cobalt-deficient pasture*

Treated pasture		Untreated pasture (lb.)
Main group (lb.)	Late lambs (lb.)	
84	48 (6 weeks old)	37½
68½	31 (3 weeks old)	83
83½	31½ (3 weeks old)	32
77½	35 (3 weeks old)	66½
66	—	30
66	—	58
77	—	45½
76½	—	55
63½	—	41½
72½	—	33
66	—	33½
53	—	62
Mean 71	—	48

Seven of the twelve animals on the untreated pasture were badly underweight and comparable to the younger lambs on the treated pasture. A complication was that all lambs in both groups harboured the tape worm, *Monieza expansa*, but faecal examination suggested that the infestation was no greater in the deficient group than in the treated group. It is well known that comparatively heavy infestations of sheep with this parasite do not appear to be harmful. The six lightest animals from the untreated pasture were taken for experiment. Three weeks later the remaining lambs on the untreated pasture were weighed again and two that had made no weight increases were added to the experiment.

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* We are indebted to Dr A. B. Stewart for suggesting the source of the first batch of cobalt-deficient lambs.

Experiment 2. The lambs used in the second experiment had been bred from Cheviot ewes in this Institute. The ewes were maintained during the later stages of pregnancy on hay and oats grown on cobalt-deficient land. The lambs were placed on the experimental diet after weaning.

Experiments 3 *and* 4. The lambs used in the third and fourth experiments were reared on a known cobalt-deficient area in the Solway region; those used in the third experiment were Blackfaces and those in the fourth experiment Border Leicester-Blackface crossbreds. The condition of the lambs in both the third and fourth groups when they arrived at the laboratory was fairly good and suggested no severe deficiency of the pasture upon which they had been reared.

The ration

Analysis of the hay used in these experiments showed that it had a cobalt content of 0·19 p.p.m. This was too high to produce deficiency, but samples analysed after it had been chopped and shaken, to remove dust and weed seeds, had a much lower content: 0·06–0·09 p.p.m. Even so it was necessary to restrict severely the quantity allowed, in order to be sure of attaining deficiency in the ration. The cobalt concentration in all constituents of the ration is given in Table 2.

Table 2. *The cobalt content of the constituents of the ration*

Constituent	Cobalt (p.p.m. in dry matter)
Chopped hay	0·06–0·09
Flaked maize	0·03
Maize-gluten feed	0·04–0·07
Steamed bone-flour	0·27
Salt	None detected
Cod-liver oil	None detected

It is believed that the concentration of cobalt in pasture grass below which deficiency will occur is 0·07 p.p.m. It was necessary, therefore, to mix the available foodstuffs in such proportions that the concentration was well below this figure. Hay was fed separately at 4 p.m. The flaked maize mixture was fed at 8 a.m. Food residues were weighed, so that the quantity of hay and maize mixture consumed was known.

The lambs in Exps. 1–3 received 200 g, and those in Exp. 4 received 150 g hay daily. Flaked maize and maize-gluten feed were mixed in the proportions of 10 : 1, and the animals were allowed as much of this mixture as they would eat up to a maximum of 1 kg daily. In practice the mixture was offered at the rate of 100 g above the previous day's food consumption until it was all eaten in a day. It was then increased by another

100 g. Twelve g of steamed bone-flour were added to the maize ration daily. The quantity of cobalt ingested, therefore, varied with the quantity of maize mixture eaten, but even at the maximum allowance of 1 kg daily the cobalt obtained from the ration was well below that from a hay diet containing 0·07 p.p.m., i.e. approximately 70 μg. The relevant figures are given in Table 3.

Table 3. *Daily intake of cobalt by the lambs in the four experiments*

(The highest content of cobalt for each foodstuff given in Table 2 was used to compute total cobalt)

Foodstuff	Cobalt p.p.m.	Cobalt Total (μg)
Hay, 200 g	0·09	18
Hay, 150 g	0·09	13
Flaked maize, 900 g	0·03	27
Maize-gluten feed, 95 g	0·07	7
Steamed bone-flour, 12 g	0·27	3
Ration (1): 200 g hay + 995 g maize mixture and 12 g steamed bone-flour		55
Ration (2): 150 g hay + 995 g maize mixture and 12 g steamed bone-flour		50

Though this ration is an unusual one, once the lambs were accustomed to it they gained reasonably satisfactorily in weight when additional cobalt was given by mouth.

Surgical procedures

Each animal in Exp. 3 was fitted with a small perspex cannula in the lower part of the duodenum, caudal to the common entrance of the bile and pancreatic ducts. Eight of the twelve animals in Exp. 4 were fitted with a rumen cannula and all animals were fitted with either a duodenal or an abomasal cannula.

The anaesthetic used was pentobarbitone (Nembutal) by intravenous injection and the usual aseptic precautions were adopted.

Rumen operations were performed in the usual way. Small-bore ebonite cannulas were used, having an internal diameter of 10 mm and an internal collar of 50 mm. The usual anatomical approach was made and the cannula was exteriorized through a stab wound approximately 1–1½ in. oral to the incision. Abomasal cannulas were of the following dimensions: internal diam. 10 mm, internal collar 30 mm. An incision 8–10 in. long was made on the right of the epigastric region parallel to the line of the last rib and separated from it by 2–3 in. The abomasum was located by the great omentum; if the organ was not lying within sight, gentle traction on the omentum brought it to the incision. The cannula was inserted into the lateral surface of the pyloric portion of the abomasum in the usual way and was exteriorized between the incision and the last rib.

In order to insert a cannula into the lower part of the duodenum an incision was made high up in the right side, beginning about 1 in. below the transverse processes of the lumbar vertebras and extending towards the mid-line of the abdomen for

8–10 in. The duodenum was found to lie transversely to the incision and was dissected free of the mesocolon for a distance of 3 in., so that no subsequent tension could draw it away from the abdominal wall. The cannula was inserted and the usual 'purse string'-suture closure of the intestinal incision was used. The shaft was then exteriorized either in front or behind the incision in the abdominal wall, depending on its position when the duodenum was returned to the abdomen.

It is unnecessary to suture either the rumen, abomasum or duodenum to the abdominal wall provided the cannula is exteriorized where no tension can be exerted on it. With the rumen and abomasum there is little danger of this happening, but the duodenum must be allowed to lie in its natural position. In all operations the simplest surgical manipulations possible were used; the fact that all animals recovered uneventfully amply justified the methods.

Sulphathiazole containing 0·2 % sodium penicillinate was insufflated around the internal orifice of the stab wound and over the peritoneal surface of adjacent viscera before closing the incision.

The animals in these experiments were transferred to their pens as soon as the operation was over, so that there was no possibility of their obtaining cobalt-rich foodstuffs. All lambs were weighed twice weekly throughout the experimental period.

Administration of cobalt

Solutions of cobalt sulphate containing either 1 or 0·1 mg cobalt in 1 ml. were administered from a 2 ml. syringe into the mouth or duodenum. Cobalt by injection was given intravenously into the jugular vein as a sterile solution of the same concentration. All doses, except in Exp. 4, were given daily.

In Exp. 4 it was found impracticable to administer cobalt by syringe into the abomasum. On account of this, 4 mg cobalt in the form of cobalt sulphate were laid on a piece of lint which was rolled into a cylinder fitting the cannula. The shafts of the abomasal and duodenal cannulas of this group of lambs were plugged with this rolled lint impregnated with cobalt, so that the inner part of the plug projected into the lumen of the organ. The plugs were changed every 4 days. In this way cobalt slowly diffused from the gauze into the organ. Control animals in this group received either no additional cobalt or 0·1 mg cobalt by mouth daily.

Analytical procedures

Livers were weighed, cut into small pieces with stainless steel scissors, placed in tared silica basins and weighed. They were dried in a stainless steel oven at 110° to constant weight. Ashing was begun over an open bunsen flame. Asbestos squares, with holes cut to fit the basins exactly, were used. Gentle heating was applied until the 'frothing stage' was over. The basins were then placed in a thermostatically controlled muffle and ashed at 500° to constant weight.

The cobalt content of the ash was determined spectrographically after chemical concentration (Mitchell, 1948; Mitchell & Scott, 1948).

Rumen contents of animals in Exp. 4 were withdrawn by suction from the rumen of each sheep with a rumen cannula in the 15th week of the experiment, as it was important to discover whether the cobalt concentration in the rumen was affected by the method of dosing. Rumen liquor (100 ml.) was withdrawn, dried to constant weight and ashed at 500°.

RESULTS

Experiment 1

The animals of this experiment were used to ascertain whether cobalt deficiency could be maintained by feeding the experimental ration and whether the ration was adequate if cobalt was given orally, and to re-investigate the claim that cobalt given by injection was ineffective in preventing deficiency.

The best and the worst of the first six lambs were placed in the group receiving cobalt by mouth; the weights of the other animals were so similar that there was little to choose between them. These animals already showed a severe degree of pining and, coming from grass, they did not take well to the experimental ration. Their appetites were very poor, except for one animal receiving cobalt by mouth. Two further animals taken later from the cobalt-deficient pasture were placed in the experiment after two deaths had occurred. The weight changes are given in Table 4.

Table 4. *Exp.* 1. *Weight changes* (*lb.*) *from initial weight of lambs after stated periods on the deficient ration*

Lamb no.	Initial wt.	Treatment	Length of period (weeks)									
			1	2	3	4	5	6	10	14	18	21
872	37½	0·1 mg cobalt by mouth daily	−3½	−5	−6½	−7½	−7½	−4½	+1	+9	+18½	+24½
874	30		−8	*	—	—	—	—	—	—	—	—
875	43		—	—	−4	−1½	+1½	+3½	+15	+23½	+25	+34
869	33½	0·1 mg cobalt intravenously daily	−1½	−4	−7½	*	—	—	—	—	—	—
871	33		−5½	−9	−13	−11½	*	—	—	—	—	—
870	33	No cobalt	−3½	−3	−7½	*	—	—	—	—	—	—
873	32		−1½	−4	−10	*	—	—	—	—	—	—
876	56		—	—	−6	−4	−6½	−4½	−3	−9	*	—

* Indicates death of the lamb.

This experiment showed that the ration unsupplemented by cobalt was not adequate for normal nutrition. The lambs receiving cobalt by injection apparently derived no benefit from it and even lamb no. 875, which was in good condition and weighed 56 lb. when placed on the ration, also failed to gain weight and ultimately died, although initially its food consumption was satisfactory. One of the animals, no. 874, receiving cobalt by mouth died soon after the experiment was begun. It was in very poor condition, weighing only 30 lb. at the beginning of the experiment, and failed to regain its appetite. Its companion, although it lost at first a considerable amount of weight, began to regain weight after 7 weeks and finally increased its initial weight by 24½ lb. in 21 weeks. From the beginning of the experiment this animal appeared brighter than the others, in spite of its loss in weight in the first few weeks.

The two replacement lambs, nos. 876 and 875, provided an interesting comparison, for the better of the two, no. 876, received no cobalt and died after 12 weeks, whereas no. 875 received cobalt by mouth and put on 34 lb. in 19 weeks.

Experiment 2

The animals consisted of home-bred lambs and the experiment ran concurrently with the first. Owing to the severity of the deficiency set up in the animals in Exp. 1, and to the excessive maize feeding, this group was used to ascertain whether a nicotinic-acid deficiency was also induced by the large quantities of maize products in the ration.

The seven animals were maintained on the ration until they were no longer gaining weight. They were then subdivided into two groups, one of which received nicotinamide in saline by intramuscular injections daily, while the other (control) received saline only by intramuscular injection. The dose of nicotinamide was 10 mg/kg body-weight.

The weight changes of these lambs are given in Table 5.

Table 5. *Exp.* 2. *Weight changes* (*lb.*) *of home-bred Cheviot lambs at stated periods on cobalt-deficient ration*

	Weight		Week					
Lamb no.	When put on ration	When injections began	1	2	3	4	5	6
730	40½	34½ (N)	−1	−½	−2½	−1½	−3½*†	—
756	27½	24½ (N)	−½	−1	−1½†	—	—	—
757	39½	39½ (N)	+1	+1	+1½	+3½	+2½	+2½
771	46	42 (N)	−½	−½	−2	−4	−3†	—
768	33½	33 (S)	+1½	+2	+4	+7	+7*	+7
772	30	29½ (S)	+1	−2	−2	−2½	—	—
773	28½	25½ (S)	0	+2½†	—	—	—	—

(N): received nicotinamide intramuscularly.
(S): received saline intramuscularly.
* Received 1 mg cobalt daily by mouth after the 5th week.
† Died before next weekly weighing.

This experiment showed that injection of nicotinamide was of no benefit to the lambs; all of them, except nos. 757 and 768, progressively became weaker and ultimately had to be killed when in a moribund condition. Injections were stopped when only four lambs were left and, instead, nos. 730 and 768 received 1 mg cobalt in solution daily by mouth, while nos. 771 and 757 were given a similar volume of saline orally. Nos. 730 and 771 both became so weak within the next week that they had to be killed, but the two surviving animals, nos. 757 and 768, were observed for the next 10 weeks. The weight changes of these lambs while receiving cobalt or saline by mouth are given in Table 6.

These lambs consumed a more satisfactory quantity of food than the lambs of Exp. 1, and there is no possibility of their subsequent decline being due solely to distaste for their food. All lambs ate sufficient quantities of food to demonstrate the fact that the diet was palatable and there was no doubt that, had they received cobalt orally, their subsequent performance would have been satisfactory.

Table 6. *Exp.* 2. *Weight changes (lb.) of two home-bred lambs, one of which received cobalt*

Lamb no.	Previous treatment	Weight when treatment changed	Week									
			1	2	3	4	5	6	7	8	9	15
757	(N)	42 (SM)	−½	+1½	+½	+½	0	−1½	−3	−2½	−7*	—
768	(S)	40 (C)	0	+1	+5	+2½	+9	+7½	+9½	+14½	+12	+16½

(N): received nicotinamide by intramuscular injection.
(SM): received saline by mouth.
* Lamb killed before the beginning of the 9th week, when it was too weak to stand.
(S): received saline by intramuscular injection.
(C): received 1 mg cobalt daily by mouth.

Experiment 3

The animals in this experiment were used to study the effect of administration of cobalt into the duodenum:

two received the ration and saline into the duodenum daily;
two received the ration and 0·1 mg cobalt into the duodenum daily;
one received the ration and 0·1 mg cobalt by mouth daily.

The Blackface lambs proved to be sturdier than the Cheviot lambs in withstanding cobalt deficiency, as is indicated by the weight changes shown in Table 7.

Table 7. *Exp.* 3. *Weight changes (lb.) of the Blackface lambs*

Lamb no.	Treatment	Initial wt.	Week								
			2	4	6	8	10	12	14	16	18
90	Saline into duodenum	34½	−9½	−13	−11	−5½	−4	−5½	−2½	−2½	−4½
92		38½	−6½	−5	−8½	−9	−7½	−10*	—	—	—
93	Cobalt into duodenum	41	−8½	−8	−8	−11½	−13*	—	—	—	—
94		34½	0	+1½	+7	+6½	+7½	+6	+6½	+3	+5
91	Cobalt by mouth	35½	−½	+1	+5	+6½	+12½	+13½	+19½	+22½	+26

* Indicates death of the lamb.

The shortest survival of any lamb in this group was 11 weeks, in contrast to the Cheviot lambs in Exps. 1 and 2, the majority of which died in 6 weeks. The longest survival in the Blackface group without cobalt was 18 weeks and this lamb was then still far from moribund, although stunted and very thin.

The peculiar part of this experiment was the behaviour of two lambs receiving cobalt into the duodenum; one behaved as though it received no cobalt at all, whereas the other appeared to derive some benefit, although it made no constant progress. The only lamb that throve was the one that received 0·1 mg cobalt daily by mouth: it gained 26 lb. in 18 weeks. An attempt was made to resuscitate lamb no. 92 by giving it 1 mg cobalt by mouth daily after the 7th week, when there was no doubt as to its deficiency; this treatment was continued for the next 3 weeks; in spite of this the animal made no improvement and ultimately died.

A study of the figures for food consumption given in Table 8 shows that the appetite was closely related to the history of the lamb, and the depressed food consumption of lamb no. 94 is a clear indication that the cobalt it received by duodenum did not have the same result as the same quantity of cobalt given by mouth to lamb no. 91.

Table 8. *Exp. 3. Food consumption (g) of Blackface lambs*

	Saline in duodenum				Cobalt in duodenum				Cobalt by mouth	
	Lamb no. 90		Lamb no. 92		Lamb no. 93		Lamb no. 94		Lamb no. 91	
Week	Hay	Maize mixture	Hay	Maize mixture	Hay	Maize mixture	Hay	Maize mixture	Hay	Maize mixture
1	100	340	50	345	110	300	210	425	380	565
2	0	170	0	1090	90	780	450	920	540	1440
3	30	100	50	1085	120	800	790	1120	330	1495
4	0	164	260	694	60	744	750	1184	680	1624
5	0	374	350	654	90	894	1140	1614	760	1844
6	200	654	600	744	460	584	1260	1864	850	2974
7	650	794	720	824	870	624	1340	2214	1030	3154
8	570	2094	550	564	1040	794	1400	2104	600	2814
9	760	1684	610	1024	780	774	1300	1714	670	3734
10	960	1234	240	1014	340	524	1050	1854	580	3684
11	960	2014	480	1374	260	200	1050	2084	620	4204
12	1050	1254	380	1424	—	—	960	1734	440	4604
13	1050	1894	100	1404	—	—	880	1322	230	5414
14	1050	1644	110	1182	—	—	1050	2014	400	5364
15	1050	1254	—	—	—	—	900	2044	250	5504
16	1050	1324	—	—	—	—	1010	1784	410	4244
17	1050	1004	—	—	—	—	1000	1964	410	4664
18	1050	1034	—	—	—	—	850	1864	570	4374

Concentration of cobalt in the liver

Values for the livers of most of the lambs of Exps. 1–3 are given in Table 9.

There was very little cobalt in the livers of lambs given no cobalt, and the figures found are lower than any other previously recorded from cobalt-deficient animals in the field. Oral dosing with 0·1 mg cobalt daily for 18 weeks or more led to substantial deposits of cobalt in the liver. Cobalt by mouth for a period of 1 or 2 weeks in animals in the last stages of deficiency led to little or no deposition of cobalt in the liver. At the rate of 1 mg daily, cobalt did not produce a large deposition in the livers, except in one animal (no. 92) which died 3 weeks after the treatment was begun.

Intravenous injection of cobalt and the introduction of cobalt into the duodenum both increased liver cobalt considerably, the former to a greater extent than the latter.

Experiment 4

The lambs in this experiment, approximately 8 months old at the beginning, were older than those in the previous experiments. They were placed on the deficient ration as soon as they arrived and none of them received any cobalt. All animals were operated upon 10–14 days later for the insertion of either an abomasal or a duodenal cannula. Rumen cannulas were placed in eight of them 2 weeks later. During this preliminary period, while the animals were becoming accustomed to the ration, some lost weight and some increased in weight; the greatest loss was 3 lb. and the greatest gain 2 lb. During the next 10 days 0·1 mg cobalt was given daily either into the abomasum or duodenum by syringe through the cannula or else by mouth. Con-

Table 9. *Concentration of cobalt in the liver of lambs in Exps.* 1–3

Lamb no.	Treatment	Cobalt: Concentration in liver ash (p.p.m.)	Cobalt: Total in liver (μg)
870	No cobalt	<0·6	<1·5
873		<0·3	<1·0
876		<0·5	<2·5
90		<0·5	<1·0
756		<0·2	<0·6
757		<0·3	<1·5
773		<0·2	<0·8
771		0·39*	1·5*
772		0·71*	2·0*
872	0·1 mg cobalt by mouth daily	14·0	79
874	0·1 mg cobalt by mouth daily (lived 10 days)	0·3	1
91	0·1 mg cobalt by mouth daily	15·0	86
768	1 mg cobalt by mouth daily	1·5	9·7
730	1 mg cobalt by mouth daily for 10 days	3·0	12
92	1 mg cobalt by mouth daily (for 3 weeks only)	22	80
869	0·1 mg cobalt daily intravenously	18	67
871	0·1 mg cobalt daily intravenously	39	89
93	0·1 mg cobalt daily into duodenum	6	22
94	0·1 mg cobalt daily into duodenum	7	30

* Additional chemical concentration allowed the exact values in the last two livers to be obtained.

siderable difficulty was experienced in dosing into the abomasum, as spillage was liable to occur; the method was accordingly changed to that described earlier in this paper (p. 180).

The initial weights and the arrangement of the experiment are given in Table 10.

Table 10. *Exp.* 4. *Weight changes (lb.) of Border Leicester-Blackface lambs*

roup no.	Lamb no.	Treatment	Operations performed	Weight: Initial	Weight: When cobalt dosing began	Week 2	4	6	8	10	12	14	16	20
(*a*)	1012	0·1 mg cobalt by mouth	R and D	41	39	+4	+9	+9½	+9	+11	+11	+16	+18½	+30½
	999		R and A	56	57½	+4	+4½	+8	+10	+12½	+13½	+21½	+27	+35
	1011		A	60½	59	+2½	+4	+7	+9	+13	+14½	+17	+20	+30½
(*b*)	993	Cobalt by abomasum	R and A	47	49½	−2	−½	+1½	+3	+6½	+10½	+13½	+19½	+37
	997		R and A	33	32½	+1½	+4	+6½	+9	+11½	+12½	+15	+19	+32
	998		A	63	65	0	−1	+2	+6	+6½	+6½	+7	+10	+29
(*c*)	995	Cobalt by duodenum	R and D	54	51	+2	+2	+4	+5	+5½	+11	+14	+14	+28½
	1010		R and D	46	45	+3	+4½	+7	+8	+12½	+15½	+19	+22½	+36
	1013		D	60	57	+½	+2	+2	+1	+8	+13	+13½	+18	+34
(*d*)	994	No cobalt	R and A	49½	45	+1	+½	+2½	+2½	+3	+2½	+2	+4½	+1½
	996		A	83	81½	+3½	+7½	+12½	+8	+8½	+9½	+10½	+12	+18½
	1014		R and D	49	46	+3½	+3	+4½	+5	+7	+8½	+9	+10½	+10

R: rumen cannula inserted. A: abomasal cannula inserted. D: duodenal cannula inserted.

The heaviest animal, no. 996, was placed in the group to receive no cobalt; this animal and no. 994 both ultimately died 8 months later and were the only casualties in this experiment. Animal no. 1014 received 5 μg cobalt by mouth daily during the last few months when it was maintained on the ration, and it survived the experiment.

The weight gains made by all animals receiving cobalt, whether by mouth, abomasum or duodenum, were all significantly greater than those made by the sheep receiving no cobalt. An apparent difference, however, was found between the oral, abomasal and duodenal groups during the first 10 weeks of the experiment and this has been analysed in detail. After 20 weeks, however, all three groups were level, and from this point onwards all behaved as though they were receiving cobalt by mouth. The detailed weight changes during the first 20 weeks are given for each individual sheep in Table 10.

When the fortnightly weight changes after 10 weeks are compared it is seen that those lambs receiving cobalt by mouth had gained 11–13 lb., those receiving cobalt by abomasum, 6½–11½ lb., those receiving cobalt by duodenum, 5½–12½ lb., and those receiving no cobalt, 3–8½ lb.

In each of the last three groups (see Table 10), one sheep made a weight gain comparable to those of group (*a*) receiving cobalt by mouth. Thus no. 996, after 6 weeks, had put on more weight than any other sheep, although receiving no cobalt. Later she lost weight and months later died. Lambs nos. 997 and 1010 similarly made weight gains equal to those of the lambs receiving cobalt by mouth. What is interesting, however, is that all other lambs, outside group *a*, nos. 993, 998, 995, 1013, 994 and 1014, made very poor weight increases, and that 10 weeks after receiving regular cobalt treatment or no treatment they had made much less progress than the lambs in group (*a*). This suggests that the cobalt introduced into the duodenum or abomasum did not have the same effect in these organs as cobalt given by mouth. But this suggestion must be treated with caution, for statistical analysis of the weight changes shows that the differences between the groups were not significant until the 12th week, when only group (*d*) receiving no cobalt differed from the others.

The concentrations of cobalt found in the rumen contents are given in Table 11.

Table 11. *Exp*. 4. *Concentration of cobalt in rumen-liquor ash of lambs*

Group no.	Lamb no.	Treatment	Cobalt in p.p.m.
(*a*)	1012	Cobalt by mouth	3·1
	999		4·4
(*b*)	993	Cobalt by abomasum	2·7
	997		1·6
(*c*)	995	Cobalt by duodenum	2·2
	1010		5·2
(*d*)	994	No cobalt	0·4
	1014		0·5

These figures show conclusively that, although cobalt was made available in the abomasum and duodenum, it attained a concentration in the rumen three or more times greater than in the rumen of the lambs receiving no supplementary cobalt. With the exception of lamb no. 1010, however, the concentration in the rumen was lower in the lambs of groups (*b*) and (*c*) than in those of group (*a*), which received cobalt by mouth.

Utilization of food by the lambs of Exp. 4. In order to ascertain whether the utilization of food was different in lambs in the different groups, the starch equivalent required for 1 lb. live-weight increase over and above that needed for maintenance was calculated for each lamb during four 5-week periods. The weight of each lamb for each period was taken as the mid-point between the weights at the beginning and end of each period. The maintenance requirements in starch equivalents for each sheep at this weight and the starch equivalents consumed were calculated from the tables given by Halnan & Garner (1944). The hay was assumed to be poor meadow hay with a starch equivalent of 22. The results of these analyses are given in Table 12.

Table 12. *Exp.* 4. *Utilization of food by lambs. Starch equivalents (lb.) consumed in excess of maintenance requirements per lb. live-weight increase*

Lamb no.	Period 1, 1st–5th week	Period 2, 6th–10th week	Period 3, 11th–15th week	Period 4, 16th–20th week	All periods, 1st–20th week
994	1·0	—*	16·3	—*	20·5
996	2·5	4·7	3·7	1·2	2·6
1014	0·8	2·6	2·7	—*	2·7
995	2·6	3·7	1·9	1·9	2·2
1010	2·3	2·4	2·8	1·9	2·3
1013	2·3	3·1	2·6	1·7	2·2
993	—*	2·4	3·6	1·8	2·6
997	1·5	2·7	2·5	1·6	2·0
998	—*	1·6	13·9	1·3	1·9
999	2·4	2·8	2·4	3·3	2·8
1011	2·2	3·4	1·8	2·1	2·6
1012	1·8	2·4	5·2	2·6	2·8

* No weight gains made, although sufficient food was eaten for them.

The results over the full 20-week period are regular for all animals except no. 994, which gained little weight in the 20-week period. In five periods certain lambs made no gains yet they ate enough food to do so. Three of these periods occurred with the cobalt-deficient lambs, nos. 994 and 1014, and the other two in the group receiving cobalt by abomasum during the first 5-week period. In one of these animals, no. 998, a second period was found in which the starch equivalents required for 1 lb. increase in weight were very high, 13·9.

These calculations, however, show that there was no constant difference in food utilization between the four groups of animals, and the weight increases were closely allied to food consumption.

DISCUSSION

The unusual nature of the ration used in these experiments was a disadvantage, for lambs coming from pasture did not eat it readily. This was most obvious with the lambs in Exp. 1. Nevertheless, when cobalt was given by mouth, most lambs made weight gains of 1–2 lb./week; although these are not optimal gains, they at least show that this ration allows growth to occur in spite of the small quantity of hay fed. One disquieting feature is that lambs in an advanced state of deficiency could not be revived when given cobalt by mouth. It is possible that this was due to changes in the bacterial flora of the rumen, and that when cobalt was again given by mouth bacteria that would otherwise take up cobalt, as found by Tosic & Mitchell (1948), had lost their ability to do so or were so reduced in numbers that the total effect was unappreciable. This hypothesis presupposes that cobalt is of use to the sheep only indirectly and that it is primarily essential for the metabolism of unknown species of rumen bacteria. In support there is the fact that the liver of one lamb (no. 92), which failed to revive after the administration of 1 mg cobalt by mouth daily for 3 weeks, contained 80 μg cobalt in contrast to that of a further lamb (no. 768), which received the same dose for a longer period and made weight gains, whose liver contained 9·7 μg, suggesting that in lamb no. 92 more cobalt was available for absorption.

The severity of the deficiency produced can be assessed by comparing the figures for total cobalt in the liver, given in Table 9, with those given by McNaught (1948–9) for naturally occurring cases of cobalt deficiency—'bush sickness'—in New Zealand. McNaught (1948–9) found the total cobalt content of the livers of lambs 5–9 months old suffering from 'bush sickness' to be 1–8 μg, compared with 10–50 μg for healthy lambs, whereas the livers of lambs 12–20 months old suffering from 'bush sickness' contained 2–7 μg. It seems probable from these figures that the cobalt available in the ration fed to our lambs was less than is present in deficient New Zealand pastures. McNaught gives the average yearly content of the dry matter of pasture grass in two deficient areas as 0·06 p.p.m., so that the average daily intake would be 60 μg, assuming that the animals eat 1 kg dry matter daily. It seems possible that the cobalt of grass is more readily available than the cobalt of hay: most of the cobalt in our ration was contained in the hay. Therefore, under our conditions, the maximum intake of cobalt, 55 μg daily, may have induced a far more severe degree of deficiency than would be encountered out of doors; the severity and rapid onset of the deficiency produced by feeding the ration, compared with the history of naturally occurring pining in Scotland, supports this hypothesis.

The fact that the two lambs of Exp. 3, which received cobalt daily into the duodenum, remained in a deficient state, even though one managed to maintain its weight over a considerable period, is convincing evidence that cobalt is not required in the intestines.

The fact that the lambs in Exp. 4 responded to the introduction of cobalt into the duodenum does not invalidate this conclusion, for it was found that the cobalt found its way into the rumens of these animals. As intravenous injections of considerable amounts of cobalt have some benefit (Ray *et al.* 1948), it is possible that cobalt can enter the rumen in the saliva or through the rumen wall. It is also possible that, in spite of the fact that the floor was slatted, the lambs may have obtained some cobalt from the faeces.

The same arguments apply to the groups with abomasal fistulas, but here oral diffusion into the rumen can be added to the possible reasons for the presence of cobalt in the rumen. The question whether free cobalt is necessary in the abomasum or the first part of the duodenum still remains to be settled. The retarded growth rate of two of the three lambs receiving cobalt by abomasum during the first 10 weeks of the experiment, however, suggests, but does not prove, that the presence of cobalt is necessary only in the rumen, reticulum and omasum. It has been long recognized that cobalt deficiency is accompanied by lack of appetite, but no careful measurements of food consumption have been made. Recently, Stewart (1951) showed that the weight changes of cobalt-deficient sheep were closely related to the appetite, and our results confirm this. In addition, they suggest that differences in the utilization of the food cannot be offered as an explanation of the deficiency syndrome, for only in one animal in which deficiency was severe was poor utilization detected on the basis of theoretical values.

As intravenous injection of cobalt does not cure cobalt deficiency, the estimation of the adequacy of the cobalt available to the animal is better judged by the concentrations in the rumen contents than by the amount present in the liver. Injection of cobalt and its introduction into the duodenum gave a substantial increase in the total cobalt in the liver of our lambs and yet these animals continued to pine. Liver cobalt is not therefore a physiological measure of cobalt adequacy and since rumen contents are easily obtained and ashed in comparison with liver samples, examination of rumen contents as a measure of the cobalt 'status' of the animal has much to recommend it.

SUMMARY

1. Cobalt deficiency in lambs has been maintained or produced by feeding a cobalt-low ration.

2. Administration of cobalt intravenously or into the duodenum at the rate of 0·1 mg daily did not cure the deficient lamb.

3. Administration of cobalt into the duodenum or abomasum in quantities greater than 0·1 mg allowed growth to occur at a rate similar to that of lambs receiving 0·1 mg cobalt daily by mouth.

4. A higher concentration of cobalt was found in the rumen of lambs receiving cobalt into the abomasum or duodenum than in those receiving no cobalt.

5. The concentration of cobalt in the liver was substantially increased by the administration of cobalt intravenously, into the duodenum or by mouth, but there was no correlation between liver cobalt and signs of deficiency.

6. The weight changes of cobalt-deficient lambs were closely related to the quantity of food consumed.

Since this paper was sent to press a report from America (Smith & Koch, 1951) has appeared to the effect that very large doses of vitamin B_{12} given by injection allow practically normal growth rates in cobalt-deficient lambs. Vitamin B_{12b} seems to be as effective as vitamin B_{12} itself.

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The Fatty Acids Present in the Rumen of Lambs Fed on a Flaked Maize Ration

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(*Received* 16 *July* 1951)

The cobalt-deficient diet introduced by Stewart (1947) contains flaked maize as this foodstuff contains very little cobalt. Experiments in which this ration was used have already been described (Phillipson & Mitchell, 1952), and examination of the rumen contents of some of the lambs involved revealed an unusual bacteriological picture (Masson, 1950, 1951), for the protozoa had disappeared from the rumen and the usual population of iodophilic cocci seen in sheep fed on diets containing starch had been largely displaced by rod-like sporulating organisms later identified as *Clostridium butyricum*. In addition, lactic acid-producing bacteria (lactobacilli) were isolated. These observations were most marked in those lambs receiving cobalt, for they ate more of the flaked maize mixture. Lambs receiving no supplementary cobalt did not show this picture to such a marked extent.

Masson's observations suggested that the acids formed as a result of fermentation would not be present in their usual proportions, and for this reason chromatographic separation of the steam-volatile acids was undertaken and the results are presented in this paper.

METHODS

Management of lambs. The eight lambs used in the experiments recorded by Phillipson & Mitchell (1952) were approximately 8 months old when they were placed on the experimental ration and were all fitted with a rumen cannula in the usual way. They were fed at 8 a.m. with the flaked maize mixture and at 4 p.m. with 150 g hay. The flaked maize mixture consisted of ten parts flaked maize and one part maize-gluten feed. To this were added 12 g steamed bone-flour daily. This ration was allowed *ad lib.* up to a maximum of 1 kg daily. Lambs nos. 999 and 1012 received 0·1 mg cobalt by mouth daily in the form of cobalt sulphate. Lambs nos. 993 and 997 received cobalt by abomasum and were fitted with abomasal cannulas for this purpose; lambs nos. 995 and 1010 received cobalt into the duodenum through duodenal cannulas, whereas lambs nos. 994 and 1014 received no supplementary cobalt. Details of the method of administration of cobalt into the abomasum and duodenum have been given by Phillipson & Mitchell (1952).

Chemical. Rumen liquor was withdrawn in the usual way and strained through muslin. Total volatile fatty acids were estimated by steam distillation and the distillates titrated with 0·05-N sodium hydroxide under carbon dioxide-free conditions. The purification and extraction of the acids for chromatographic separation were as described by Elsden (1946). Buffered columns were used to separate the acids as described by Moyle, Baldwin & Scarisbrick (1948). Column 1 was used which separates acetic, propionic and butyric acids quantitatively. Acids higher in series than butyric pass through rapidly without separating and can be collected and titrated as one fraction. They were closely followed by the wave of butyric acid, and sometimes a complete separation of butyric from the higher acids was not obtained. About 0·25–0·5 m-equiv. of acids in 5 ml. chloroform-butanol solvent were placed on the column.

Lactic acid was determined according to the methods of Friedemann & Graeser (1933); glucose, by the copper-reduction method of Somogyi (1945) and pH by the use of a glass electrode.

RESULTS

Preliminary investigations in which total volatile fatty acids only were determined showed that the concentration of these acids was rather lower than would be expected from the high starch content of the ration. Concentrations of volatile fatty acids of 1·41–3·65 m-equiv./100 ml. were found in the morning 1 h after feeding and the highest values found ranged from 6·76 to 11·72 m-equiv./100 ml. Hourly samples were drawn from each of the eight lambs from 9 a.m. to 5 p.m. and the concentration exceeded 10 m-equiv./100 ml. in only two samples.

It was clear from the analysis of these samples that a partly volatile acid was present since the titre of the distillates did not return to the blank value by the third 50 ml. portion. Lactic acid is known to occur in the rumen under certain circumstances, and direct estimations revealed the presence of high concentrations in two lambs, nos. 997 and 999, and of appreciable concentrations in lambs nos. 995 and 1014, as is illustrated in Fig. 1.

Further investigations on the remaining four lambs, nos. 1012, 993, 1010 and 994, showed that high concentrations, 7·0 m-equiv./100 ml., were reached in two of them, nos. 1012 and 1010, a concentration exceeding 0·50 m-equiv./100 ml. was attained in no. 993 and traces were present in one lamb, no. 994, which was a cobalt-deficient animal with a correspondingly poor appetite. The pH of the rumen liquor, given in Table 1, was exceptionally low.

One of the lambs, no. 993, was selected in order to examine the composition of the steam-volatile acids. Two samples were taken, one at 12 noon and one at 5 p.m., so

Table 1. pH *and concentration of lactic acid at different times of the day in the rumen liquor of lambs fed on a ration containing a high proportion of flaked maize*

	Lamb no.							
	1012		993		1010		994	
Time	pH	Lactic acid (m-equiv./100 ml.)	pH	Lactic acid (m-equiv./100 ml.)	pH	Lactic acid (m-equiv./100 ml.)	pH	Lactic acid (m-equiv./100 ml.)
9 a.m.	5·6	0·37	4·5	0·40	5·1	0·15	6·1	0·17
12 noon	4·8	2·65	4·5	4·97	4·6	6·75	6·6	0·30
3 p.m.	4·6	7·25	4·3	5·25	4·5	7·00	6·3	0·60
6 p.m.	4·7	4·25	4·9	2·75	4·6	4·12	6·1	0·22
10 p.m.	5·6	2·12	5·7	0·22	5·2	1·74	6·9	0·16

that the peak of fermentation and a later stage could be compared. The results are given in Table 2 and show that a high proportion of propionic acid was present, particularly at midday when it exceeded that of acetic acid.

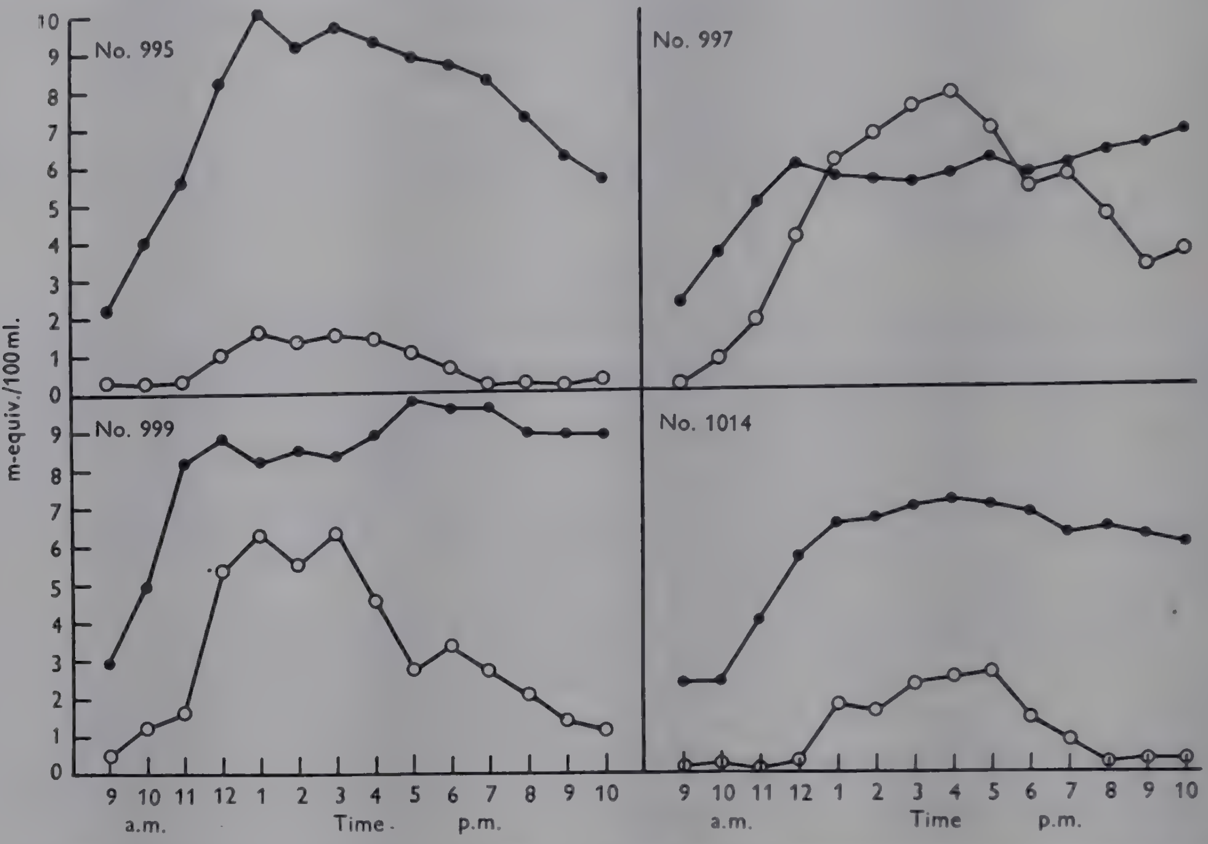

Fig. 1. Changes found in the total volatile acid and lactic acid concentrations in rumen liquor from four lambs throughout a 12 h period. The lambs received the maize mixture at 8 a.m. and hay at 4 p.m. ●——●, total volatile acids; ○——○, lactic acid.

Table 2. *Concentrations of fatty acids in the rumen of lamb no.* 993 *fed on the maize ration*

	12 noon		5 p.m.	
Acid	m-equiv./ 100 ml.	Percentage of total	m-equiv./ 100 ml.	Percentage of total
Acetic	1·50	37	2·11	45
Propionic	1·95	48	1·83	39
Butyric	0·49	12	0·56	12
Higher acids	0·12	3	0·19	4

The low proportion of butyric acid was surprising on account of the presence of considerable numbers of *Cl. butyricum*, an organism known to produce acetic and butyric acids in vitro under certain conditions. In order to confirm the low proportion present in the rumen, further samples were withdrawn at 2.30 p.m. from each of the eight lambs, and the results from them were similar except that the proportion of propionic acid, although still considerable, was not so marked as in the previous samples shown in Table 2. The proportion of butyric acid, however, as shown in Table 3, was small in all lambs except no. 1014. These results left no doubt that the acetic : propionic ratio was narrower than is usually found in sheep fed on other rations.

The peculiar results found for lambs nos. 1014 and 994 may not have been due to the inadequate cobalt in the ration; neither lamb ate as much of the flaked maize mixture as the lambs that received cobalt, and this in itself was likely to influence the result. The amount of food consumed by each lamb on the day the samples were withdrawn is given in Table 3.

The food intake was consistent for any particular period, and although the appetite of the deficient lambs decreased with time, the decrease was slow. Weight gains by lambs receiving adequate cobalt ranged from 28½ to 37 lb. over a 20-week period (Phillipson & Mitchell, 1952).

Two lambs, nos. 1012 and 1014, were selected for further work. Lamb no. 1014 was given 0·005 mg cobalt by mouth daily in order to prevent acute symptoms of deficiency setting in, since her companion, no. 994, was in poor condition and later died. The ration given to no. 1012 was reduced so as to equal as far as possible that of no. 1014. A comparison of the concentrations of lactic and volatile acids in these two lambs was made 1 month later and little difference between them was found. The lactic-acid concentrations in no. 1012 were now very small although still greater than in no. 1014. The data are given in Table 4.

A significant difference, however, was found after chromatographic separation of the steam-volatile acids, the proportion of propionic acid being greater in no. 1012 than in no. 1014. The proportion in both lambs was now less than previously, although the food eaten by no. 1014 had not changed in quantity. There was a large difference between the weights of the two lambs, however; no. 1012 weighed 73·5 lb. and no. 1014, 53 lb. at this time. Forty-two days later further samples were withdrawn from these lambs and the volatile acids again partitioned. The results of both sets of analyses are given in Table 4.

In the earlier analyses an increase followed by a decrease in the proportion of propionic acid was found in lamb no. 1012. The second set of analyses, although less complete, shows the same increase in both lambs. The proportions of propionic acid in lamb no. 1012 were, however, less in the second set than in the first, and the difference between the two lambs seemed to be disappearing.

The result of introducing an aqueous solution of 100 g glucose into the rumens of these lambs was investigated. No food was given in the morning after the first sample was drawn, and a sample was drawn before, and at intervals after, the introduction of glucose into the rumen. Glucose disappeared rapidly from the rumens of both lambs, although the concentration attained in no. 1012 was far greater than that found in no. 1014. The fermentation appeared to pass rapidly to the lactic-acid stage and slowly to the volatile-acid stage, as can be seen from Fig. 2. This was more marked in no. 1012 than in no. 1014, for the concentration of lactate formed in this lamb was greater and fermentation more prolonged than in no. 1014, whereas a change in the fatty acids was not seen till 4 h after the administration of glucose. The increase in propionic acid in no. 1014, however, although more rapid and more pronounced, was insignificant compared with the concentration of lactate formed and disappearing.

Table 3. *Concentrations and percentage composition of the volatile fatty acids in rumen samples of lambs taken 6½ h after feeding a ration containing a high proportion of flaked maize*

	Lamb no.															
	1012		999		995		1010		993		997		994		1014	
Acid	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%	m-equiv./ 100 ml.	%
Acetic	2·93	51	4·94	57	3·50	60	4·26	52	5·07	63	3·25	63	3·24	68	4·01	59
Propionic	2·41	42	3·03	35	2·04	35	3·19	39	2·49	31	1·49	29	0·29	6	1·56	23
Butyric	0·29	5	0·52	6	0·17	3	0·57	7	0·32	4	0·31	6	0·38	8	0·95	14
Higher acids	0·06	1	0·09	1	0·12	2	0·17	2	0·16	2	0·10	2	0·81	17	0·27	4
Food consumption of lambs on the day the samples were drawn																
Hay (g)	150		150		150		150		150		130		50		150	
Flaked maize mixture (g)	790		1000		690		840		1000		780		470		410	

Table 4. *Concentrations of lactic acid and volatile acids in the rumens of lambs nos.* 1012 *and* 1014, 30 *and* 72 *days after the food eaten by no.* 1012 *had been restricted*

			After 30 days					After 72 days	
Lamb no.	Acid		9 a.m.	12 noon	3 p.m.	6 p.m.	10 p.m.	11 a.m.	3.45 p.m.
1012	Lactic (m-equiv./100 ml.)		0·15	0·31	0·28	0·16	0·12	0·48	0·15
	Total volatile (m-equiv./100 ml.)		4·08	5·78	6·23	4·79	5·39	10·7	10·5
	Acetic	(As percentage of total)	68	60	60	61	60	67	65
	Propionic		16	26	30	31	29	18	21
	Butyric		12	9	7	5	6	10	8
	Higher acids		5	4	4	3	4	4	4
1014	Lactic (m-equiv./100 ml.)		0·12	0·10	0·16	0·17	0·15	0·15	0·14
	Total volatile (m-equiv./100 ml.)		5·50	6·68	7·13	7·06	7·49	11·3	12·2
	Acetic	(As percentage of total)	72	73	71	74	69	77	71
	Propionic		18	18	19	17	19	14	19
	Butyric		8	6	7	6	8	6	6
	Higher acids		2	3	3	3	3	2	3

A further experiment in which lactate was introduced into the rumens of the two lambs confirmed the results obtained with glucose. The concentration of lactate found after dosing was greater in no. 1012 than in no. 1014 and the rate at which it disappeared was slower.

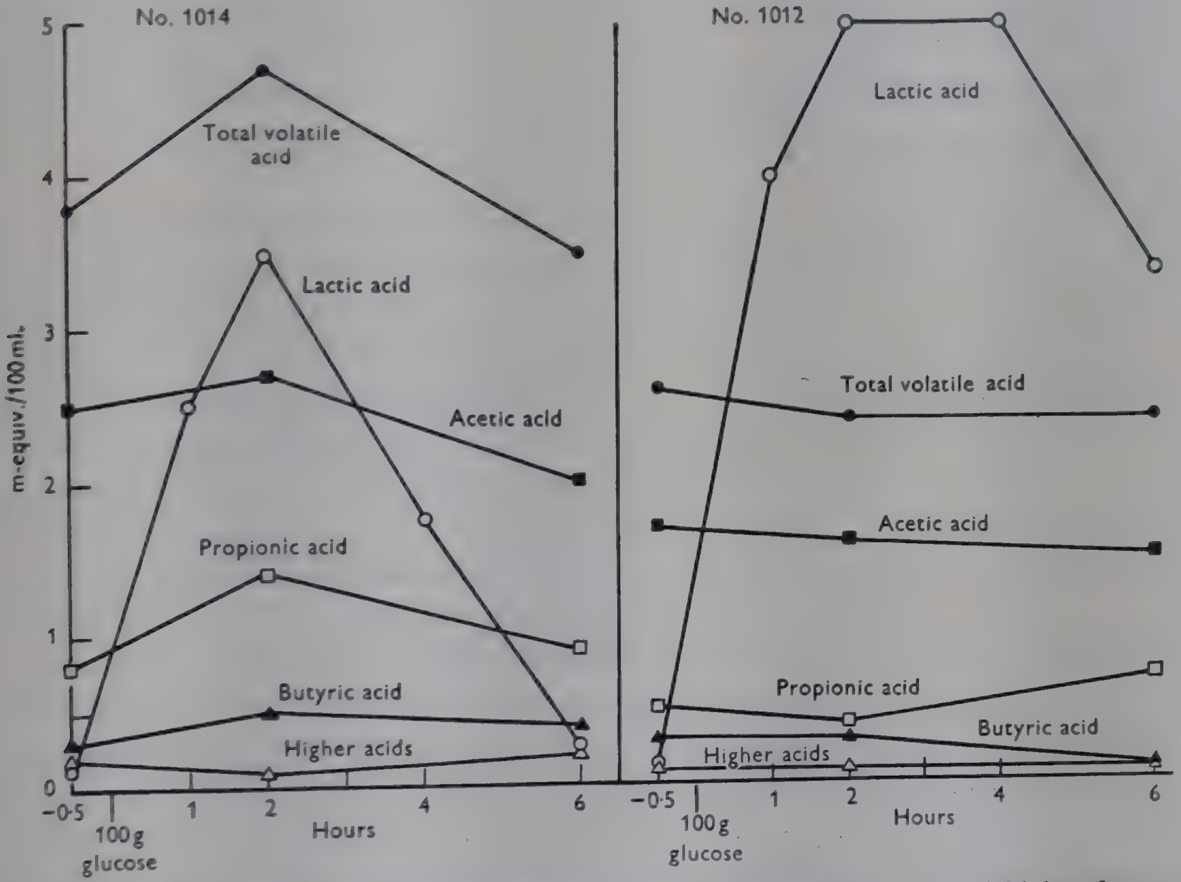

Fig. 2. Changes observed in the concentrations of acetic, propionic, butyric and higher fatty acids and lactic acid after the administration of 100 g glucose in solution into the rumens of two lambs.

DISCUSSION

The two most striking features of these analyses are the high concentrations of lactic acid in the lambs on the full flaked maize ration and the high proportion of the total volatile fatty acid represented by propionic acid. Turner & Hodgetts (quoted by Masson, 1951) and Reid (personal communication) have found high concentrations of lactic acid in the rumens of sheep receiving food containing wheat, and Reid and his colleagues have also shown that the proportion of propionic acid to acetic acid was unusually high. The present observations in which flaked maize was the source of starch agree well, therefore, with those of the Australian workers. This is a different picture from that usually found in the rumen, for under most circumstances little, if any, lactic acid is present (Phillipson, 1942), and propionic acid represents less than 20 % of the total fatty acid (Elsden, 1945; Schambye & Phillipson, 1949; Kiddle, Marshall & Phillipson, 1951), except when rations of 'wheaten chaff' (wheat cut immediately after the flowering stage and dried) and lucerne hay are fed, when values as high as 27 % have been recorded (Gray & Pilgrim, 1951). Lactic acid has previously been found in quantity only when mangolds were fed or when glucose was introduced into the rumen (Phillipson & McAnally, 1942; Elsden, 1945).

In spite of the high lactate concentrations, those of total volatile fatty acids were lower than is usual in fully fed sheep. The total volatile fatty-acid concentration in the rumen of sheep in this Institute fed the usual indoor rations or maintained only on hay varies usually between the levels of 60 and 130 mmol. In these lambs the range was lower and on only two occasions exceeded 100 mmol. Lactate is unstable in the rumen of normally fed sheep, and on account of this seldom accumulates in any quantity; if it does, as for an instance in sheep eating mangolds or after the introduction of glucose into the rumen, the concentration decreases nearly as rapidly as it had previously risen.

Elsden (1945) has isolated propionic-acid bacteria from the rumen of sheep, and Johns (1951*a–c*) has studied in detail the fermentation of lactate by *Veillonella gazogenes*, which he found to be numerous in the rumen. Johns found that the ratio of acetic acid to propionic acid was influenced by the carbon-dioxide tension in the medium, and suggested a scheme for the fermentation of lactate which terminated by the decarboxylation of succinate to propionic acid and carbon dioxide. Elsden & Sypesteyn (1950) have also observed this reaction, using washed-cell suspensions of rumen bacteria.

The fermentation of starch in the rumen of these lambs appears to have taken place in at least two stages in a manner postulated by Elsden (1945); the first stage leading to the formation of lactic acid and the second being the further fermentation of lactic acid with production of volatile fatty acids of which propionic acid is prominent. *Cl. butyricum* in pure culture is known to form acetic and butyric acids in an acid medium; it was surprising, therefore, that such small quantities of butyric acid were present in the rumen. In media in which the pH is buffered between 6 and 7 the presence of bicarbonate encourages lactic-acid formation by this organism, although it is possible that the important factor here is the presence of bicarbonate rather than the stabilization of the pH. *Cl. butyricum*, therefore, may be responsible for some of the lactic acid produced. The presence of lactobacilli, however, provides an additional reason for the large concentrations of lactate present. Most of these organisms ferment only sugars, but according to Sherman (1937) *Streptococcus bovis*, normally present in the alimentary tract of the ox, hydrolyses starch and produces lactic acid from glucose and a variety of other sugars. Both these organisms, therefore, could be concerned with the production of the high concentrations of lactic acid observed.

The introduction of glucose in the rumens of lambs nos. 1012 and 1014 caused the immediate production of high concentrations of lactic acid, but the changes in the concentrations of acetic and propionic acids were insignificant in comparison. It appears, therefore, that the first stage of lactic-acid fermentation proceeded at a greater rate than the subsequent fermentation of lactate to acetic and propionic acids, and whereas the high proportion of propionic acid is positive evidence of lactate fermentation as studied by Elsden (1945) and Johns (1951*a*), the speed of this fermentation appeared to be slower than that of the initial starch fermentation.

The low pH found in the rumen of these lambs favours rapid absorption of short-chain fatty acids and according to Danielli, Hitchcock, Marshall & Phillipson (1945) the longer the hydrocarbon chain the more rapid this absorption, so that the low concentration of total volatile acids may be due partly to the rapid absorption of butyric, and to a less extent of propionic, acid. Even so, the rapid production of any acid in the rumen is associated with an increase in its concentration, and I have found no evidence of a significant increase in concentration of butyric acid in these experiments; indeed the reverse occurred in lamb no. 1012 in the series of analyses given in Table 4.

These investigations were undertaken in order to determine whether any differences could be detected between the lambs receiving cobalt and those receiving no supplementary cobalt. Reference to Table 3 shows that there was a marked difference in the fatty-acid picture of no. 994 and the propionic acid present in no. 1014 was proportionately less and butyric acid proportionately greater than in the other lambs. These differences, however, may have been due to the differences in the quantity of food rather than to inadequate cobalt. The striking feature of the analyses made on no. 994 is not only the exceptionally low proportion of propionic acid but the exceptionally high proportion of higher acids, which suggests rapid deamination of protein according to Shazly & Synge (1950). This lamb ultimately died. The subsequent comparison of lambs nos. 1012 and 1014 failed to reveal any real difference between them, and no conclusions can be drawn concerning the influence of cobalt in the results obtained.

SUMMARY

1. Lambs fed on a ration of 150 g hay and a mixture of flaked maize and maize-gluten feed in the proportions of 10 : 1 to a maximum of 1 kg daily, developed unusually acid conditions in the rumen although their weight gains were adequate if supplemented by cobalt.

2. Lactic acid appeared in the rumen in greater concentration than is usually found, and values exceeding 7 m-equiv./100 ml. were observed. The concentration of acetic acid was lower, and the concentration of propionic acid greater, than is usual, so that the ratio acetic : propionic acid was unusually narrow.

3. Fermentation of glucose in the rumen of two lambs resulted in the rapid accumulation of lactic acid. The subsequent changes in the concentrations of acetic and propionic acids were insignificant in comparison.

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Some Observations on the Diet of Jamaican Children, with Particular Reference to Liver Disease

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(*Received* 18 *July* 1951)

The object of this communication is to describe the inadequate diet of Jamaican children suffering from liver disease.

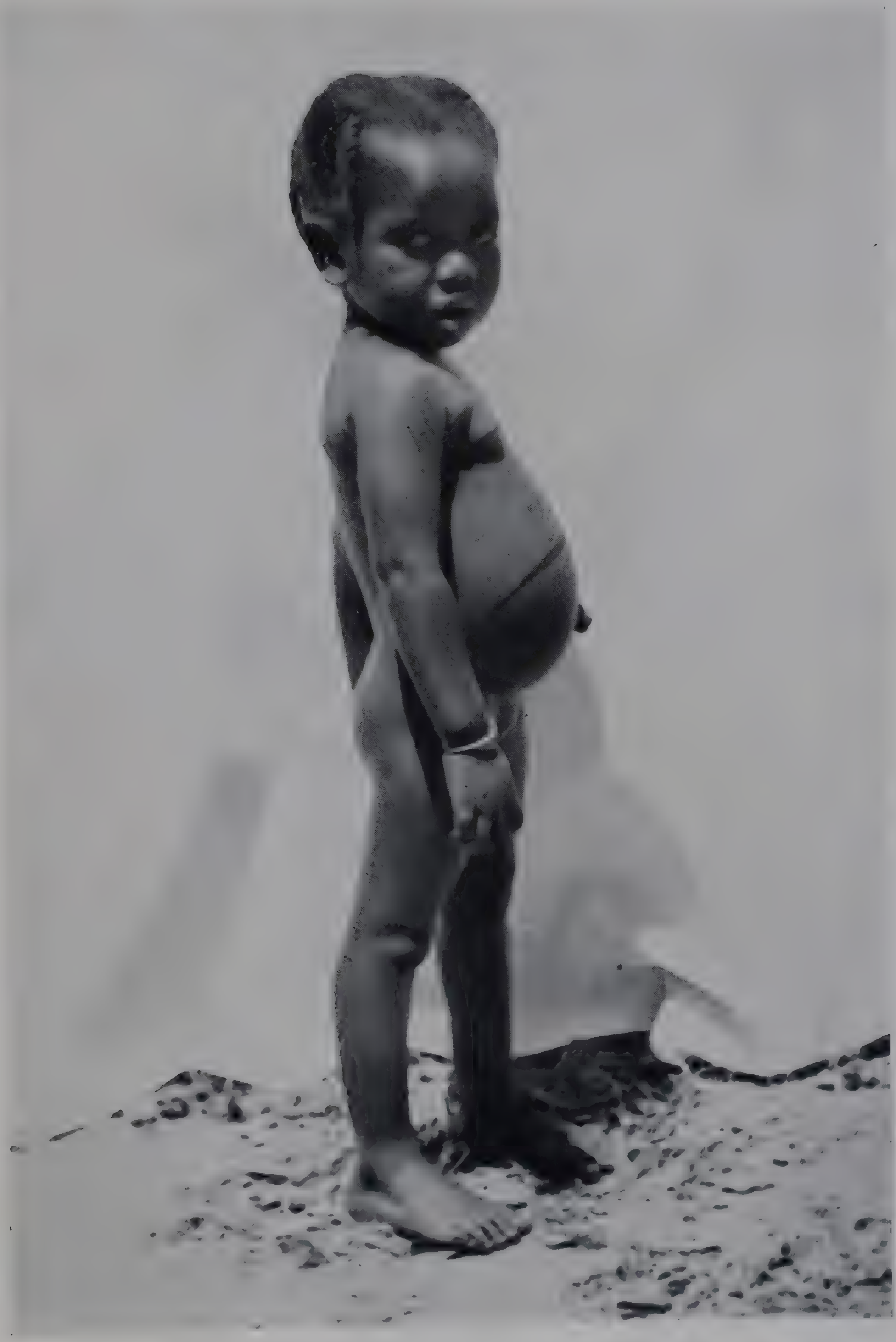
EXPERIMENTAL

Clinical material studied. During the past 15 months, 115 cases of liver disease have been investigated in this Department by Hill (1951) and Hill & Rhodes (1951). Ninety liver biopsies were performed on fifty-eight of the cases by the needle technique and, in addition, ten post-mortem specimens were examined. Though the majority of these patients looked chubby and well nourished, all had an enlarged liver (Pl. 1) and some had enlargement of the spleen with ascites and, occasionally, oedema of the legs. If ascites was present it refilled rapidly after paracentesis abdominis. None of the patients had malaria or any other acute or chronic infection or infestation with parasites.

The liver sections showed a continuously developing histological picture which we have termed 'serous hepatosis'; this picture, commencing with a serous exudation within the perisinusoidal spaces of Disse, passed on to the laying down of an eosinophilic coagulum which subsequently became fibrosed to give a final appearance of what is usually known as cirrhosis (Hill, 1951). Fatty metamorphosis of the liver as described by Waterlow (1948) was found only in five cases; these however showed, in addition, the fundamental pathological lesions described above. Approximately 30 % of the total number of children, clinically and histologically, had cirrhosis of the liver with fibrosis, hyperplasia and distortion of lobular architecture; these belonged to an older age group and had longer medical histories.

Dietary investigation. The diet of each of the children suffering from liver disease was inquired into during this investigation, but the food intake of ten children was studied in more detail and compared with that of five healthy controls. Their ages varied from 9 months to 8 years, the majority being between 9 months and 3 years. Eight were male. The dietary measurements were made in the children's homes by

KATERINA RHODES. Diet of Jamaican children with liver disease *Plate* 1



daily visits over a period of 4–7 days. If measurements were conducted for less than 7 days it was because the daily dietary pattern was being repeated without any change whatsoever. All food was weighed in the dry, uncooked state. Co-operation was obtained from the mother or guardian in all cases by making her understand the purpose of the inquiry, thus eliminating errors due to false statements. The same utensils as were used in the preparation of meals, for instance cups and spoons, were used in indicating quantities. The food values were calculated from tables compiled by Platt (1945), Nicholls (1945), Wooster & Blanck (1949), and the daily averages obtained were compared with the average requirements recommended by the (U.S.A.) National Research Council (1948).

Physical examination. Of the five healthy children, two were seen at the local Child Welfare Clinic and three in the surgical Children's Ward of Kingston Public Hospital. Physical examination revealed no abnormality in these cases. Particular attention was directed towards any enlargement of the liver or spleen or the presence of deficiency disease. The larger group of ten children had obvious liver disease. In all ten the liver was hard and enlarged, two had enlargement of the spleen and three had ascites with oedema. Liver biopsies performed in seven of these cases revealed various stages of the developing hepatic fibrosis described above, i.e. serous exudation followed by an eosinophilic coagulum followed by fibrosis. None of the fifteen cases described were being breast fed at the time of investigation. All came from comparable social strata and were resident in the urban area of Kingston.

RESULTS

In all figures and tables, nos. 1–5 represent control healthy children and nos. 6–15 the sick children with liver disease.

Table 1 shows age, sex, weight and height and the average daily protein, carbohydrate, fat and calorie intake for each of the fifteen children.

Dietary differences between sick and healthy children

General. The daily average protein and fat intake of the children with liver disease was invariably much lower than that of the healthy group. For example, child no. 2 of the control group aged 3 years had a daily protein intake of 34·2 g, whereas children, cases nos. 11 and 13, of the same age had protein intakes of 10 and 19·5 g, respectively. The calculated carbohydrate and calorie intake did not differ in the two groups, except for cases nos. 6 and 8 which were grossly deficient. These children had severe hepatic fibrosis with splenomegaly and did not thrive. It is true that the children with liver disease received fewer calories than those in the control group, but the difference is not sufficiently marked to be significant. The last line in Table 1 shows the number of different food items taken in one week. The difference between the two groups is marked: the diet taken by the sick children was monotonous, whereas that of the healthy group, taking into account the age of each individual child concerned, was very much more varied.

Protein intake. Fig. 1 gives a diagrammatic representation of the total protein intake and of the proportion of animal protein in the diet of the two groups. The

Table 1. *Tabulation of dietary intake of children with reference to age, sex, height and weight*

	Healthy children					Children with liver disease									
Case no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Age	6½ y	3 y	9 m	1 y 4 m	1 y 8 m	2 y	8 y	4 y	10 m	2 y 7 m	3 y	5 y	3 y	2 y	10 m
Sex	M.	F.	M.	M.	F.	M.	F.	M.	M.	M.	M.	F.	F.	F.	F.
Weight (kg)	18	13¾	8·2	11·5	10	10·8	16	10·8	9	12·25	8	9·5	14	9	5·4
Height (cm)	109	—	72·5	—	77	75	112	85	67·5	82	77·5	94	92·5	86	62·5
Daily protein intake (g)	37·8	34·2	21·7	31·3	27·5	5·2	11·7	8·7	10·3	14·5	10	11·7	19·5	12·7	10
Daily fat intake (g)	31·9	23·2	20	26·9	41·7	4·3	4·7	24·1	5·6	8·7	4·5	4·7	16·7	4·3	10
Daily carbohydrate intake (g)	230·2	124·2	168	115·1	175·4	67·4	162	64·2	147·6	210·6	153·8	162	142·7	225	157
Daily calorie intake (Cal.)	1474	828	986	902	1268	345	774	539	718	1026	730	774	854	1035	791
Weekly no. of food items	28	16	6	8	24	6	6	5	5	17	5	6	8	9	5

y = years; m = months.

protein standard is calculated as 3·5 g protein/kg body-weight. The animal-protein standard is taken as two-thirds of the total protein ((U.S.A.) National Research Council, 1948). Control cases nos. 1–5 had a total protein intake ranging from 60 to 78 % of the normal, with an arithmetic mean of 72·5 %. These were the healthy children. The children with liver disease (nos. 6–15) had a total protein intake not higher than 52·5 % of the normal, ranging from 13 to 52·5 % with an arithmetic mean of 32·6 %.

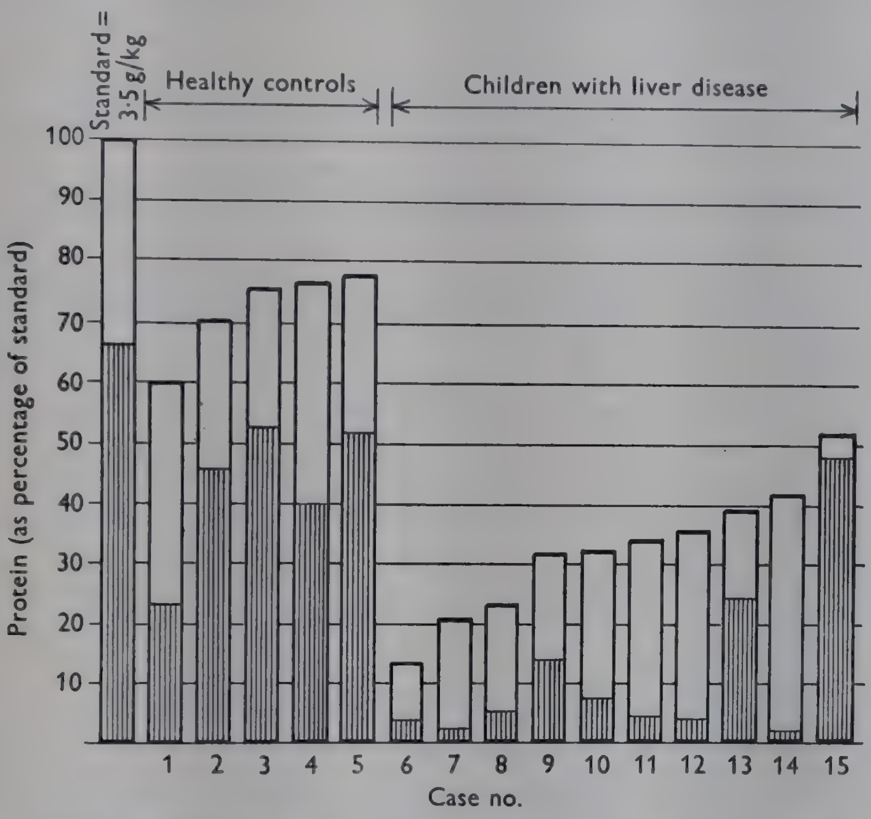


Fig. 1. Daily protein intake, and proportion of animal protein, ▥, in the diets of the sick and healthy children, expressed as percentages of standards (see top of page).

It is obvious from the diagram that the proportion of animal protein in the diets of the children with liver disease was likewise much smaller than for the healthy children with the exception of cases nos. 14 and 15 which, nevertheless, showed great deficiency compared with the National Research Council standard. It may be suggested that the protein requirements adopted as standard are too high, since even the more adequately fed and healthy children had a deficiency of from 22 to 40 %. A standard for the British West Indies has not yet been compiled, but whatever standard requirements are adopted, the difference in the protein intake of the two groups of children will still be significant.

Source of protein. Table 2 presents the different articles of food eaten by the fifteen children. It shows that eight of the ten with liver disease derived their animal protein from a single source only, seven from condensed milk and one from fresh cow's milk. Of the remaining cases (nos. 10 and 13) one had only a small quantity of beef in addition to condensed milk, and the other a small amount of beef and egg. Of the healthy children, one, no. 3, a child 9 months old, obtained all available protein from condensed milk alone. The remaining four children had a combination of sources

Table 2. *Detailed diet of children under investigation*

(Total weekly quantities in g)

	Case no.														
	1 (6½ y)	2 (3 y)	3 (9m)	4 (1 y 4m)	5 (1 y 8m)	6 (2 y)	7 (8 y)	8 (4 y)	9 (10m)	10 (2y 7m)	11 (3 y)	12 (5 y)	13 (3 y)	14 (2 y)	15 (10m)
Coco-nut milk	406	—	—	—	20	—	—	—	—	—	—	—	—	—	—
Tea	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cocoa	3	28	—	—	—	—	—	21	—	—	—	—	—	—	—
Ovaltine	7	—	42	—	49	—	—	—	—	—	—	—	—	—	—
Lime	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Grape-fruit	—	—	—	—	268	—	—	—	—	57	—	—	—	—	—
Orange	160	1050	—	105	156	—	—	—	170	—	—	—	—	—	227
Tomato	74	—	—	—	75	—	—	—	146	—	—	—	—	—	—
Lettuce	15	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Callalu	—	40	—	—	48	—	—	—	—	—	—	—	—	—	—
Cabbage	37	—	—	—	24	—	—	—	—	32	—	—	—	—	—
Carrot	96	340	—	—	242	—	—	—	—	90	—	—	—	—	115
Turnip	30	44	—	—	—	—	—	—	—	—	—	—	—	—	—
Chocho	—	—	—	—	90	—	—	—	—	120	—	—	—	—	—
Congo peas	90	—	—	—	—	—	—	—	—	12	—	—	—	—	—
Yellow yam	439	—	—	—	168	—	453	—	—	90	—	453	—	175	—

Irish potato	157	240	—	—	—	—	—	—	—	240	—	—	—	—	—
Plantain	120	—	—	—	32	—	—	—	—	—	—	—	—	58	—
Ripe banana	—	400	—	—	500	—	—	—	—	52	—	—	—	—	—
Green banana	—	—	—	—	—	—	600	—	—	401	400	600	—	116	—
Rice	311	112	—	—	112	—	—	—	—	303	—	—	87	58	—
Whole-wheat flour	33	—	284	—	—	—	—	—	—	—	—	—	—	—	—
Arrowroot	—	—	—	—	114	70	—	—	—	—	—	—	—	—	227
Pearl barley	—	—	—	126	—	—	—	—	—	—	—	—	273	—	—
Patent barley	—	—	40	—	—	—	—	—	—	—	—	—	—	—	—
Quaker oats	—	—	—	136	—	—	—	—	—	62	—	—	—	—	—
Cornmeal	—	—	45	—	—	105	538	—	454	27	538	538	—	532	—
Brown bread	—	392	—	784	—	—	—	—	—	—	—	—	—	—	—
White bread	1001	—	—	24	316	203	56	504	—	309	56	50	218	336	—
White sugar	—	70	—	70	—	—	—	—	—	—	—	—	—	—	—
Brown sugar	126	—	256	—	243	219	538	91	454	277	538	538	350	833	455
Coco-nut oil	94	—	—	—	16	—	—	—	—	—	—	—	—	—	—
Margarine	45	—	—	—	12	—	—	140	—	30	—	—	—	—	—
Butter	—	35	—	49	50	16	—	—	—	—	—	—	—	3	—
Lobster	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Salt fish	37	—	—	—	25	—	—	—	—	—	—	—	—	—	—
Fresh fish	—	25	—	—	60	—	—	—	—	—	—	—	—	—	—
Corned beef	52	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fresh beef	184	100	—	—	60	—	—	—	—	30	—	—	98	—	—
Egg	40	54	—	94	—	—	—	—	—	—	—	—	294	—	—
Cow's milk (fresh)	—	1589	—	2778	1702	297	—	—	—	30	—	—	—	—	—
Condensed milk	424	339	1299	—	563	—	113	210	397	202	113	113	399	59	795

y=years; m=months.

* A very small quantity which could not be measured.

including cow's and condensed milk, eggs, beef and fish. The healthy children averaged 3·2 different sources of animal protein and the sick ones 1·3. There was also a difference in the types of vegetable protein taken by the two groups. In the cases with liver disease, nos. 6–15, the pattern seems to have been one of white bread, maize meal and green banana, whereas control cases, nos. 1–5, had more varied sources with greater protein content, such as brown bread, whole-wheat flour, rice, vegetables and fruit.

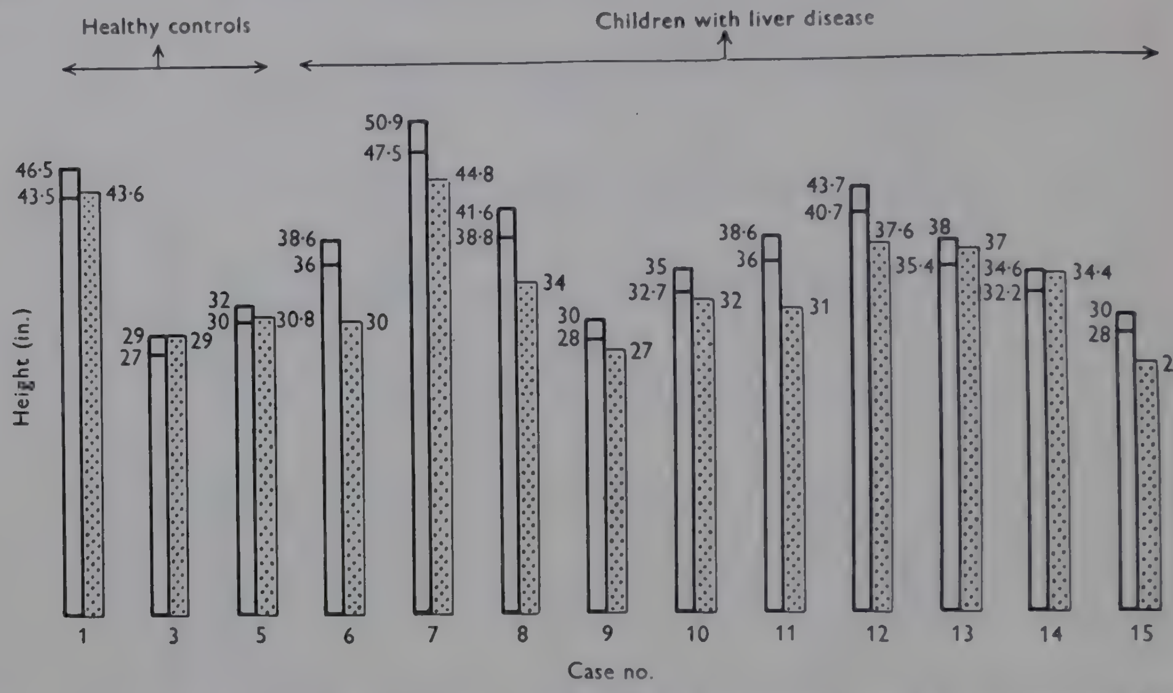


Fig. 2. Comparison of actual with standard heights (Holt & McIntosh, 1941) for the sick and healthy children. The white column indicates the standard height with the upper and lower limits of normal variation; the dotted column the actual height of the children.

Heights of the children

When the height of each child was compared with average heights for similar age groups (Holt & McIntosh, 1941) it was found that most of the children with liver disease were markedly stunted. Fig. 2 shows the relationship of actual heights to standard heights. Unfortunately, the data for two healthy children were lost. It was noted at the time that all five were not below average height. Our findings concerning the heights of normal children are in agreement with figures obtained in a survey of 2636 girls and 2279 boys in the Kingston area aged from 4 to 15 years, between the years 1935 and 1943 (Cumper, 1950). Children nos. 6–15 were markedly below average, with the exception of nos. 13 and 14, which were asymptomatic cases of hepatic enlargement found on routine examination, that is to say relatively mild cases.

DISCUSSION

On the whole, it may be said that the healthy children and most of the children with liver disease discussed here, appeared to be well nourished; however those with liver disease were not so tall as the healthy controls. It is known that the biological value of animal protein is superior to that of protein of vegetable origin, and a mixture of proteins has a higher value than single proteins (Nicholls, 1945). The group of children with liver disease had a very low intake both of total protein and of first-class protein of animal origin. However, their monotonous diet supplied them with adequate calories, which were for the greater part derived from sugar and refined cereal foods. The cereal taken was usually a stereotyped one, used day after day and month after month, and it was in the majority of instances yellow degerminated maize meal. The main, and mostly the only, source of first-class protein was in the form of sweetened condensed milk. The Jamaican mother adds sugar to the diluted condensed milk to make it more palatable, thus further increasing the carbohydrate proportion in the milk. This disproportion of carbohydrate to protein may well play a part in the development of liver disease. Furthermore, this monotonous diet may create a deficiency because the protein portion of the cereal used may not be composed of properly balanced essential amino-acids. The child with liver disease, subsisting on a diet that is inadequate, possibly at first compensates by retardation of growth. Later the liver may fail to synthesize certain enzymes of protein origin (Waterlow, 1950). If these enzymes themselves are concerned with protein metabolism, then a vicious circle of dysfunction in protein metabolism may commence. At present it is not known whether the lack of single or multiple amino-acids or the disproportion of protein to carbohydrate intake or perhaps a combination of these factors contributes to the development of liver disease. There may be other aetiological factors at work. It has been found, however, that a diet rich in protein favourably influences the course of the disease. A balanced diet rich in animal protein has met with success in many cases of liver disease. In this series, the time necessary for improvement to become obvious was from 2 to 4 months and sometimes even longer. For this reason, a protein concentrate (Ventriculin, Parke, Davis and Co.) was tried with excellent results and a much more speedy response; improvement, sometimes dramatic, began to take place within 4–21 days. Ventriculin and high-protein foods such as milk and liver had been tried previously in the various liver diseases in children with partial or complete success, reported by Williams (1933) in West Africa, Gillman & Gillman (1945) in South Africa, Carvalho (1947) in Rio de Janeiro, Altmann (1948) in South Africa, Waterlow (1948) in the British West Indies, Trowell (1949) in West Africa, Thomson (1950) in Malaya and Hill (1950) in Baghdad. Parenteral administration of whole blood or serum by Pieraerts (1949, 1950) in the Belgian Congo, Achar (1950) in India and Van der Sar (1950, personal communication) in Curacao was also of value. These observations are in favour of the conception that one of the underlying causes of liver disease in the tropics is a dietary one.

SUMMARY

1. The diets of ten Jamaican children with liver disease were compared with those of five apparently healthy Jamaican children.

2. Compared with healthy controls the children with liver disease had a diet deficient in total protein and in animal protein.

3. Their diet contained, in general, only one source of animal protein, in the majority of instances sweetened condensed milk.

4. The diet lacked variety.
5. It is suggested that one of the underlying causes of liver disease in Jamaican children is dietary.

I am indebted to Professor K. R. Hill for helpful criticism and advice in the preparation of this paper, to Messrs Parke, Davis and Co. (Detroit) for supplying the Ventriculin and to Mrs H. Mills for typing this script.

EXPLANATION OF PLATE

Pl. 1. Jamaican child with liver disease.

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PROCEEDINGS OF THE NUTRITION SOCIETY

SEVENTIETH SCIENTIFIC MEETING
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE
6 OCTOBER 1951

THE FEEDING OF NORMAL AND PREMATURE INFANTS

Chairman: Professor S. J. COWELL, *University College Hospital Medical School, London, W.C. 1*

General Principles of Infant Feeding

By S. Graham, *Department of Child Health, University of Glasgow*

The subject of infant feeding is one well worn by previous discussion, but there is always something to be gained by reviewing the accepted facts and presenting again the controversial matter. My task is to deal with the subject in a general way, and as there are papers by other speakers on breast-feeding (Waller, 1952) and artificial feeding (Smellie, 1952) I shall do my best to avoid anything more than a casual reference to these particular aspects.

One accepts the fact that human milk taken by the infant from the mother's breast is the ideal food for the human infant: this needs restating, particularly at the present time, because it has recently been questioned and figures have been published showing that small infants fed on a modified cow's-milk mixture with a high protein content, can achieve more rapid gains in weight than with breast milk (Gordon, Levine & McNamara, 1947). That this is so can be accepted, but it is salutary to remind ourselves that good nutrition cannot be determined merely by a gain in weight. Indeed, except for the very small premature infant in the first weeks of life, a too rapid gain may be disadvantageous: overweight infants succumb to infections more frequently than those who are of average weight for age.

Cause of failure to gain weight

There is no doubt, I think, that in the past artificial feeding has been made unnecessarily complicated, and this has resulted in a tendency to recommend some proprietary food, telling the mother to follow the instructions on the label. If the infant thrives all is well, but if he fails to thrive, owing to some congenital abnormality or infection, that particular brand of food is blamed and another one tried. This often heralds the onset of a long series of changes usually with a falling caloric intake and consequent failure to gain in weight. The end result of such a regime is a wasted infant who is regarded as a 'feeding problem' with the implication that the mother was unable to find a food to 'suit the baby'.

As a working hypothesis, it may be suggested that an infant fails to gain in weight for one or more of three reasons: some congenital abnormality, an infection, or underfeeding. We are not concerned in this discussion with the first two, which belong to the realm of clinical medicine. As far as the feeding is concerned, the usual mistake is not of giving the wrong food but of giving it in amounts inadequate for the needs of the infant. The percentages of fat, carbohydrate and protein are relatively unimportant and 'percentage feeding' fostered for so long by the German school, has now been discarded. A similar attitude can be taken to artificial breast milk and humanized milk. Cow's milk cannot be humanized, and this process as far as infant feeding is concerned is wasteful of time and money. The important point is that the milk in whatever form it is given to the infant must be free from pathogenic organisms. Therefore all milk must be sterilized, preferably by heat (boiling). This applies even to breast milk that is exhausted from the mother and kept in a breast-milk bank. Here I would like to pay tribute to the Ministry of Health who in wartime provided a subsidized dried milk for infant feeding. Among the various factors which in the last 10 years have contributed to the fall in infantile mortality, the provision of an ample supply of cheap, sterile, vitaminized dried milk must rank very high.

Taking a broad view of infant feeding there are three main aspects for consideration: (1) the quality of food, (2) the quantity of food, (3) the accessories (minerals and vitamins); and two subsidiary ones: (1) the technique of breast and artificial feeding, and (2) the prevention of infection. The technique of infant feeding may be held to include the emotional and psychological factors that in some infants loom so large, and to the practising physician the prevention of infection in the small infant presents many problems as yet unsolved.

Calorie requirements

I should like to deal specially with the quantity of food necessary to make an infant thrive. Its importance has already been stressed. To determine it, some system is required and some unit of energy necessary. For years now, the Calorie (large) has been used, and although from time to time there have been other suggestions made, none has proved popular. Without some method of expressing the amount of energy required, infant feeding becomes a hit or miss affair. To quote Graham Lusk (1919) 'the so-called "scientific feeding" of infants is unworthy of the name unless the caloric requirement is carefully considered. From lack of this knowledge babies are frequently systematically starved.' The older child can express his feelings of satisfaction or hunger in different ways, but the underfed infant has the same method as one with colic from overfeeding.

The quantity of food required obviously varies with the weight of the infant, that is, with his age. Normally, the infant's weight is doubled in 6 months and trebled in a year. This rate of growth is far beyond anything that occurs at any other period of life and is the rational explanation of why deficiency diseases such as rickets and iron-deficiency anaemia appear so readily in the 1st year of life.

Infants require food for four purposes. These and the energy requirements/kg/day for each are as follows (Fleming, 1923–4):

Basal requirement	55 Cal.
Growth	5 Cal.
Loss in excreta	5 Cal.
Muscular activity	35 Cal.

The first three of these are capable of reasonably accurate measurement; the fourth, muscular activity, is a guess, but small infants unable to walk or stand or even sit up unsupported do not vary in this respect as much as older children, and an allowance of 35 Cal./kg/24 h is generally accepted as adequate.

The sum of these four requirements is 100 Cal. One should aim therefore at giving the healthy infant 100 Cal./kg/day certainly for the first 9 months of life, because the basal metabolic rate remains unchanged throughout this period. Having arrived at this figure, it only remains to convert the calories into terms of food for infants of varying ages. To do this, the following information is required:

Average birth weight	3·2 kg
Gain/month for first 6 months	0·6 kg
Gain/month for second 6 months	0·5 kg

One oz. milk (30 ml.) yields 20 Cal.
One rounded teaspoonful of sugar (5 g) yields 20 Cal.

An average healthy infant aged 3 months weighs 5 kg (3·2 kg + 1·8 kg) and requires 500 Cal. or in terms of milk 25 oz. daily. If he is fed 4-hourly, with five feeds daily at 6 a.m., 10 a.m., 2 p.m., 6 p.m., 10 p.m., this works out at 4 oz. milk and one teaspoonful of sugar in a feed. If this amount is taken by the infant and retained (i.e. there is no diarrhoea, vomiting or fever) and absorption is adequate (as it should be in healthy infants), he will thrive. The only important cause of failure to thrive, apart from disease processes, is underfeeding.

Requirements of the undernourished infant

I should like very briefly to make one more point that is of the greatest importance in the feeding of the undernourished infant. It seems fairly obvious that the calorie requirements will be the same for undernourished as for heavier infants as long as the basal metabolic rate remains unchanged. The fall in it does not occur unless the infant is 65 % or less of his expected weight.

In practice this means that a 3-month-old infant who weighs 4 kg instead of 5 kg (i.e. who is 80 % of his expected weight) requires as much food as if he weighed 5 kg. He has as much active metabolic tissue, but 1 kg less of inactive metabolic tissue in the form of fat and water. Too often the underweight infant is fed less than the healthy infant of the same age, the argument being that because he is small he should be fed less. Infants therefore should be fed according to their expected weight (that is, according to their age) rather than their actual weight. Prescribing an adequate feed for a healthy infant is thus simplified. One needs only to know his age to determine his expected weight.

In very marasmic infants, when the weight is below 65 % of the expected weight, and the basal requirements fall, less food will of course be required and allowance can be made for this by a simple calculation.

SUMMARY

The importance of the caloric intake in infant feeding is stressed and an outline of the reasoning behind the determination of the amount of food required at various ages is given. This applies equally to breast- and bottle-fed infants.

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The Importance of Breast Feeding

By H. K. WALLER, *British Hospital for Mothers and Babies, Woolwich, London, S.E.* 18

The implication in the title of my paper is that breast feeding has an advantage over artificial feeding. That indeed is my belief, but The Nutrition Society will not want belief but proof; and frankly I cannot bring forward any likely to convince it. The rate of infant mortality has been falling steadily in this country during the past 50 years; the period being notable for a great improvement in the safety of bottle feeding. There is no evidence that I know to show the reduction in the death rate of babies owes anything to an increase in the number breast fed.

For such proof as there is of the importance of breast feeding we have to rely on carefully planned statistical surveys. Often quoted is one carried out by Grulee and his co-workers in Chicago between 1924 and 1929 (Grulee, Sanford & Schwartz, 1935). The mortality rate of the 20,000 babies they reviewed was but 11/1000; and among the wholly breast fed only 1·6/1000. These figures are so low that when published they seemed quite inapplicable to this country. Morbidity is much more difficult to assess, but they claimed that the incidence of infection was directly related to the method of feeding: 37 % in the breast fed, 64 % in the artificially fed. I will only quote one example of work in this country, where epidemic enteritis has always been a special risk to young bottle-fed babies. Despite the improvement in safety already mentioned, in a series of 216 cases of infantile gastro-enteritis, Gairdner (1945) found recently a death rate of 61 % when breast feeding lasted for less than a month, and 28 % if it had been prolonged beyond that time. I chose that example because in clinical practice there is ample, one might say daily, evidence that the ability to accommodate itself to artificial milk is least in the newborn and becomes progressively greater with the passage of time. So much so that by the age of 6 months there are not many who will not thrive on cow's milk. They thrive, that is to say, when judged by the standards which we usually employ, such as records of weight and height, the age at which balance in the upright position is attained, the date and order of dentition and so on. In discussing these standards with parents, doctors, or at least the prudent among us, always allow a wide margin for the 'normal' in the individual. Here, however, an even greater reservation seems to me needed.

It is generally accepted that the differences in the composition of the milks of mammalian species have been evolved in accordance with the particular pattern of growth best suited to the young. Thus the young seal, which is suckled for a matter of 3 weeks only, increases in weight from about 30 to 90 lb. mainly by the deposition of fat. The mother's milk is said to contain 43 % of it. Now surely the most impressive feature of the human baby's growth is the rapid development of the central nervous system. The brain reaches nearly two-thirds of adult size by the age of 2 years, and almost its full size by 7 years. I know of no nutritional studies on the bearing of infant feeding on this phenomenon. But I sometimes wonder whether we may not dismiss too lightly the possibility that the composition of human milk may be specially related to the rapid growth of nervous tissue; whether, in fact, the functional efficiency of the adult brain may not suffer from the substitution of bovine for human milk in the early stage of its growth. And if this seems too absurdly far fetched to put forward in seriousness, may I remind the reader how often we used to see in babies suffering from rickets—a deficiency disease—quite grave disturbances of the central nervous system; spasmus nutans, laryngismus, tetany and convulsions. Though I have no figures to support me, it is my impression that convulsions are considerably more common in bottle-fed than in breast-fed babies, and I think all paediatricians are agreed that the effect of convulsions in infancy can be very serious. (I hardly dare suggest that some part of the psychiatric therapy, which seems to be becoming an almost indispensable part of the up-bringing of children, may one day be replaced by dietetics, should we get to know more about the relationship at which I have hinted.)

A recent inquiry (Joint Committee of the Royal College of Obstetricians and Gynaecologists and the Population Investigation Committee, 1948) revealed that 43 % of 13,000 babies were artificially fed by the age of 8 weeks, and that another 12 % were partly so fed. We may thus suspect that soon after that age half the 13,000 were wholly bottle fed. It is clear that if the main risk of this method of feeding is to be reduced we need to know why it is that breast feeding ceases so early for so many. Unfortunately this large survey provides no answer, and the same holds good for many others of smaller size. For example, a careful review by Robinson (1943) in Liverpool of 1100 failures of lactation, showed no explanation of 40 %.

It is a problem I have been studying for a number of years and have a few observations which perhaps are worth offering. In dairy science it is well established that the size of the milk yield of animals, as well as its security, depend on the degree to which the udder is regularly emptied. If this is true of the mammary gland in women, as I think it is, then one finds in practice that it is often not recognized. There are many reasons for incomplete emptying at the start of lactation, especially the first lactation. The baby's contribution to emptying the breasts may be hampered by anatomical defects of the nipple. The initial yield may be greater than even a large infant can utilize. The newborn may be frail and weakly as, for example, when it is born prematurely; or it may be reluctant to feed because of illness or birth injury. And lastly the breasts' function of expelling the milk may be interfered with. Compared with these a true insufficiency (hypogalactia) seems to be uncommon, though it is often advanced as a main reason why many women cannot succeed. In Fig. 1 the yield is shown of a series of 400 women as they passed through the hospital wards. They were equally divided between primiparas and multiparas. Seventy-eight %, or almost four-fifths of the 400, were yielding 20 oz. milk, or more, on the 10th day after delivery. If they were representative, the figure suggests that failure arises from an inability to maintain an originally satisfactory yield.

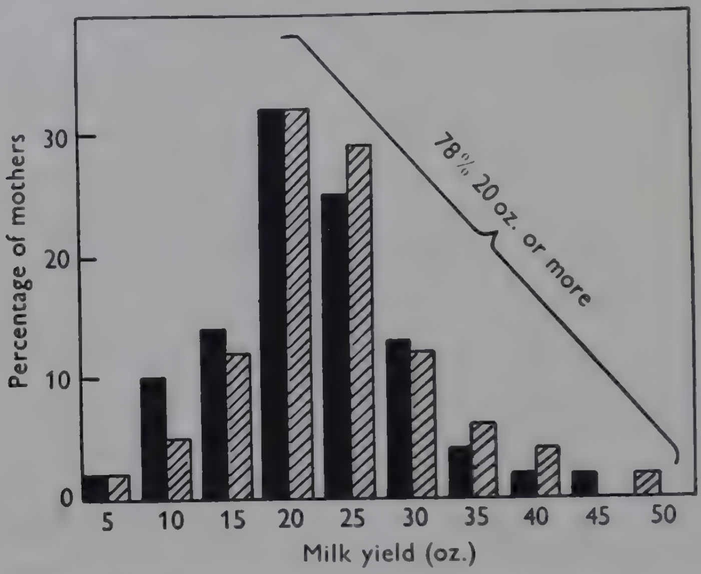

Fig. 1. Milk yield of a series of 400 women (200 primiparas and 200 multiparas) on the 10th day post-partum. Solid columns, primiparas; hatched columns, multiparas.

A nipple that is tied to the deep structure of the breast and cannot be drawn far into the baby's mouth is a great obstacle to emptying, for when squeezed between the jaws it retracts towards the breast. The lactiferous sinuses are then out of reach. This defect is easily found by pinching just behind the nipple's base, and if detected early enough in pregnancy can be corrected, or much improved, by the use of the shield illustrated (Pl. 1). It is worn under a brassiere which keeps the nipple pushed through the hole in the base of the shield and gradually stretches its attachment. Some degree of defect in one or both nipples is found in about 40 % of our primigravidas. Figs. 2 and 3 show differences in the degree to which the newborn drains the breasts. In Fig. 2 emptying is almost complete, and lactation may be considered secure. In Fig. 3 there is a gross disproportion between the amount of milk produced and what the child can take. Only 43 % of the yield is withdrawn. Here, unless the residual milk had been removed by hand, this woman's lactation would probably soon have ceased. She represents a large class who tell us they had too much milk at first, but that it left them when they got up. They are commonly accused of being unwilling to breast feed.

Fig. 4 illustrates the weekly yield of a young woman who gave birth to a baby weighing 2 lb. 2 oz. (960 g) at the 28th week of her first pregnancy. It demonstrates how easily the yield can be preserved by the regular manual removal of the milk. The baby was not strong enough to feed direct from the breast until the 8th week. Soon afterwards it obtained all it needed, and only the excess was expressed. Later still demand and supply balanced. We teach all our patients during pregnancy the use of their hands for this purpose. There is not time to teach them between delivery and the start of milk secretion.

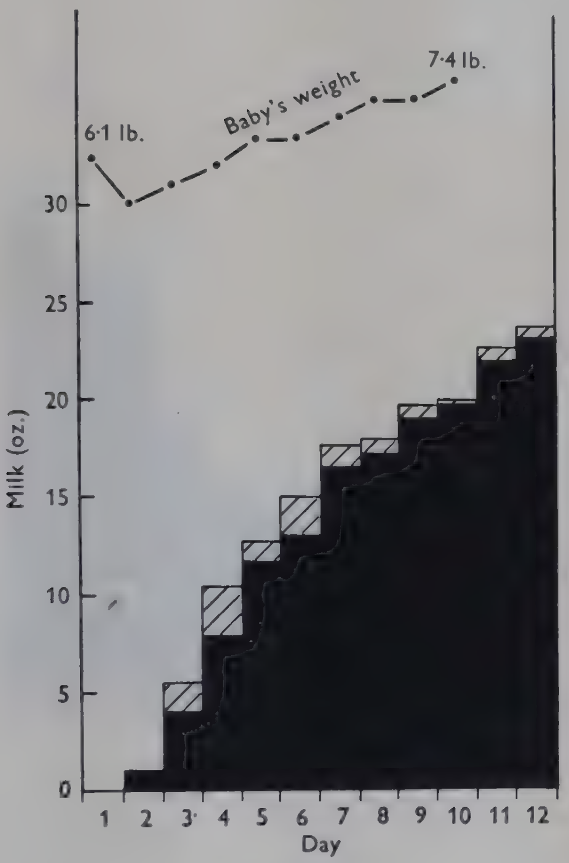

Fig. 2

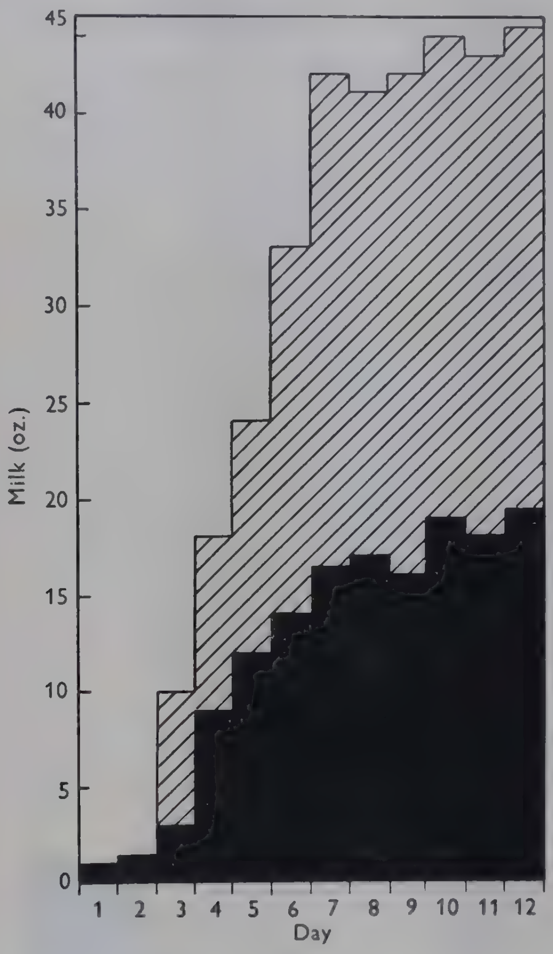

Fig. 3

Fig. 2. Almost complete emptying of the breast by newborn baby. Solid area, milk removed by baby; hatched area, residual milk removed by hand. Total yield in 12 days, 167 oz., removed by baby 156 oz., ratio 93 %.

Fig. 3. Partial emptying of the breast by newborn baby. Solid area, milk removed by baby; hatched area, residual milk removed by hand. Total yield 341 oz., removed by baby 147 oz., ratio 43 %. Birth weight of baby 6·2 lb.

Interference with the reflex expulsion of milk is often seen when the secretion starts rapidly and is copious. The breasts are then apt to become very overloaded. They may indeed be lumpy and oedematous, and then outflow is obstructed. This is difficult to deal with and is a common source of failure; for if the state of high milk tension persists for several days it is followed by resorption of milk and the changes of involution. If the danger is recognized early, it can be controlled by the judicious use of one of the preparations of oestrogen. Fig. 5 shows an example. Pain as an inhibitor of reflex expulsion has been mentioned, and there can be no question that suckling when the surface of the nipples has been broken can cause very acute pain. The remedy is not to allow the baby to suckle, and to remove the milk by hand until the breach in the surface is healed. This simple measure would by itself prevent many women from weaning their babies; it would also lessen the high incidence of acute lactational mastitis and breast abscess which give breast feeding such a bad reputation.

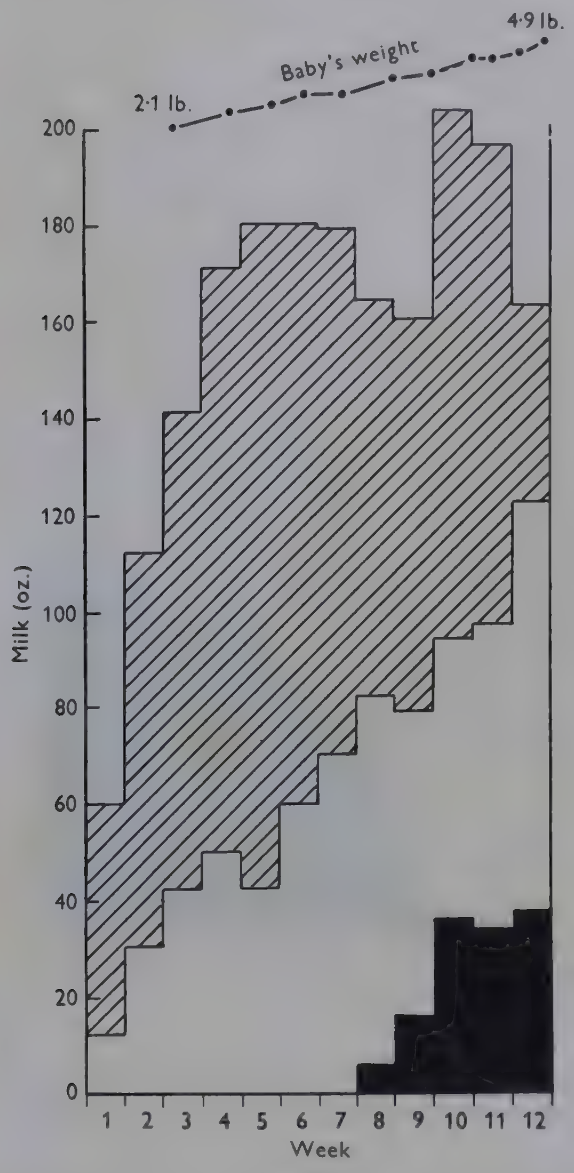

Fig. 4

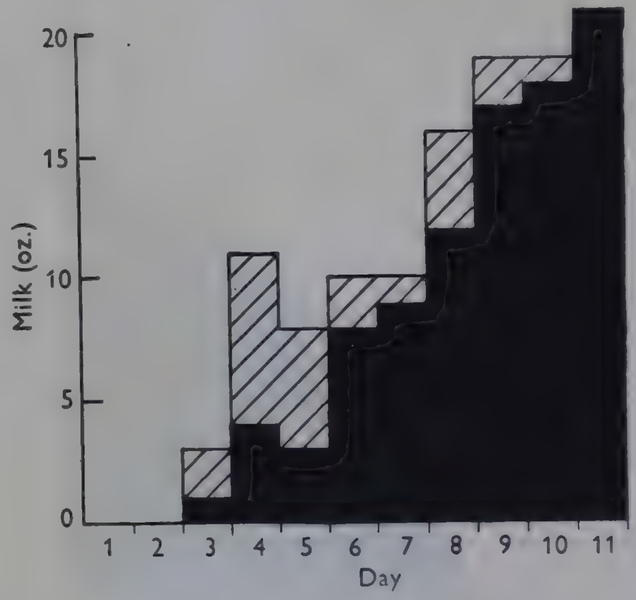

Fig. 5

Fig. 4. Weekly milk yield of mother of premature baby. Solid area, milk removed by baby; white area, milk removed by hand and given to baby by bottle; hatched area, milk removed by hand but not given to baby.

Fig. 5. Control of breast engorgement on 3rd day by administration of stilboestrol and manual expression. Solid area, milk removed by baby; hatched area, residual milk removed by hand.

It has been the teaching at our hospital that, far from being a simple physiological function, the start of breast feeding is a difficult task for many women. To obtain adequate drainage of the breasts from the very outset calls for skilled attention to detail; with its aid it is interesting to see how often women are enabled to succeed who have failed previously with a succession of babies. Another striking fact is that, granted the right help, unwillingness to breast feed seems to be uncommon. Several

series which we have followed beyond the lying-in period have shown it is not difficult for between 70 and 80 % to continue to breast feed for 6 months and longer.

EXPLANATION OF PLATE

Pl. 1. Breast shield to be worn during pregnancy for correcting defective nipples.

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Composition of Human Milk and Factors Affecting It

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The composition of breast milk is of interest primarily because it is the recipe of the most satisfactory food for a baby. It is also to some extent a measure of the mother's nutrition and an indication of the mother's food requirements. Its variation may help to reveal how milk is formed.

The secretion of the mammary gland varies during the course of lactation, starting during pregnancy and the first days after delivery as colostrum, changing to milk which again alters in composition with the passage of days and weeks. Eventually the secretion of full milk is succeeded by a secretion resembling colostrum. This has been called regression milk. After the first acceleration on the 3rd or 4th day the rate of secretion of milk is almost constant from hour to hour and day to day with a slow increase of about 1–1½ oz. on the day's total in the course of a week to meet the baby's growing needs. The removal of milk, partly brought about by the mechanism of the baby's mouth, is aided by an expulsive mechanism within the breast. This, the draught reflex, may be impaired by emotion. The degree of emptying depends not only on the activity of the baby but on the vigour and timing of the draught and there is no clear end-point. The milk that is not removed at one feed is believed to be added to the subsequent secretion. As the fat globules of milk tend to remain in the gland, the fore-milk contains little fat, the hind-milk much more and the milk obtained at a feed or at a sampling may vary much in fat content.

Nitrogenous substances. The protein of breast secretion normally decreases rapidly in the first days and then more slowly throughout lactation; the first viscous material may contain as much as 10 % or more protein; towards the end of the 1st week the percentage has usually fallen to less than 2, and after months of lactation may be less than 1. The content of protein is affected very little, if at all, by diet, as is shown by the agreement between the figures relating to peacetime United States (Bell, 1928) and wartime Britain (Kon & Mawson, 1950). In the very first days the fall is more rapid where the output is greater. In the cow, the protein content of colostrum is reduced when milking is begun before calving, and a similar effect of antenatal expression may explain the low values found in some estimations of protein in colostrum after delivery. The protein content of the first secretion removed may be above that of the serum, and similarly the globulin concentration, judged by the titre of the antibodies, may be above that of the serum. Despite extensive investigation there is no evidence that these antibodies are absorbed unaltered by healthy babies (Boorman, Dodd & Gunther, unpublished).

The protein of mature milk contains 0·7–0·8 g lactalbumin and lactoglobulin in 100 ml., rather more than cow's milk (0·5 g/100 ml.), but the casein (0·4–0·5 g/100 ml.) is much less than that of cow's milk (3·0 g/100 ml.) (Nelson, 1950). The non-protein nitrogen is slightly less in human than in cow's milk, but the free amino-acids are about the same in amounts and proportions in both.

Much emphasis has been laid in the past on the amino-acid composition of the protein of breast milk, probably because it was thought that protein is absorbed from the gut only after being broken down to amino-acids. Williamson (1944) and Block & Bolling (1950) have shown that the proportions of the acids in human and in cow's milk are almost alike although, as is well known, the total protein content is higher in cow's milk. Even the subdivisions, casein, lactalbumin and lactoglobulin in both kinds of milk all resemble one another in their amino-acid proportions. There is, however, some doubt about the sulphur-containing amino-acids. It has been maintained, on the basis of some estimations of cystine, that there is more of it in human than in cow's milk, and this has been advanced as an advantage of human over cow's milk. The reported values for the cystine content of human milk differ, ranging from 1·8 to 4·0 g/16 g nitrogen. The methionine ranges from 2·2 to 2·5 g/16 g nitrogen, and the corresponding values for cystine and methionine in cow's milk are, respectively, 0·8 and 3·4 g/16 g nitrogen (Block & Bolling, 1950). Cystine is said to be difficult to estimate, and the recent analyses imply that the similarities between human and cow's milk extend to include the proportions of sulphur-containing amino-acids. Block & Bolling (1950) conclude that 'the often expressed opinion that breast milk is superior for the *protein* nutrition of the human infant must be based either on differences in the non-protein amino-acid fraction or on teleological reasoning'. The evidence of a large survey (Douglas, 1950) shows, on the other hand, that in England the breast-fed child has specific advantages. When allowance had even been made for birth weight and body-weight, the breast-fed baby was shown to walk on average some 10 days earlier than the bottle-fed and to have received an immunity to measles. It is tempting to conclude from the protection against measles that breast milk gives babies a greater store of γ-globulin; possibly the globulins of milk are broken down by digestion sufficiently to lose their antibody pattern and yet retain some linkages that assist the baby to reconstruct human γ-globulin. A similar suggestion has been put forward by Mellander (1945) who has shown that the products of tryptic digestion of human and cow's milk differ; the human milk retains a phospho-peptone of glutamic acid, isoleucine and serine containing organically bound phosphorus. Mellander suggests that this complex, if absorbed intact, might explain in part the breast-fed child's freedom from rickets. If the structure of a protein leaves its mark on the nitrogenous substances absorbed from the gut, the amino-acid composition of milk proteins represents only part of the proteins' nutritive value, and the debate on the relative merits of breast and cow's milk enters a new phase.

Fats. Since the breast cannot be emptied in the first days of lactation, the fat content of early milk is hard to determine. At the end of the 1st week the percentage in milk collected over 24 h is about 3, and it tends to increase slightly in the next days. The fat content during the rest of lactation appears to run no consistent course (Nims, Macy, Hunscher & Brown, 1932), and the factors controlling it are little understood. The percentage of fat in 24 h samples is little affected by the fat in the diet in normal circumstances, but there is some evidence that starvation or a diet containing very large amounts of fat may cause a big reduction in the output of milk and an increase in the percentage of fat (Ružičić, 1934). Moderate supplements of fat given to women on a low-fat diet had no significant effect on the percentage of fat in the day's secretion of milk in one investigation, although there was some slight rise in fat content some 6–12 h after the fat had been eaten (Gunther & Stanier, 1951). There is a diurnal variation in the fat content for which there is at present no explanation (Planchu & Rendu, 1911; Gunther & Stanier, 1949); it is unaffected by the mother's intake of calories or fat. The variation may be as much as from 2 to 4 % fat, the highest content occurring about noon. This variation is so great that the mechanism controlling it must be the principal one determining the content of fat in milk. Although the percentage of fat is apparently independent of the fat intake, some of the fat is believed to be derived from ingested fat (Bendix, 1898); it is also believed that ingested fat may alter the action of enzymes concerned with fat synthesis (Hilditch, 1947).

The composition of the fats of breast milk is remarkable for the absence, or very low content, of the short-chain saturated fatty acids. The C_{18} acids constitute about 57 % of the total, about one-half being unsaturated (Hilditch & Meara, 1944; Brown & Orians, 1946). The proportion of unsaturated acids of the diethenoid series and the amounts of C_{20-22} acids are characteristic of breast-milk fat; in this it resembles human depot fat and is more like vegetable-oil fat than butter.

Sugar. Lactose, the sugar characteristic of breast activity, is present in slightly lower amounts in colostrum (about 5 %) than in milk (about 6·5 %), the value remaining almost constant after the 10th day. Lactose is not better than sucrose or glucose in the artificial feeding of infants and we are still without an explanation of why this particular sugar is present in milk. One can only speculate that the formation of lactose is in some way involved in the processes of secretion of the fat and protein.

Vitamins. The very numerous estimations of vitamins made by Kon & Mawson (1950) have provided a fairly clear picture of the variations in their secretion. The amounts of vitamin A and of carotenoids are directly related to the fat content of milk, although there is also an inverse relation, the concentration being less in the fat where the fat content of the milk is high. Kon, Mawson & Thompson (1944) have shown that the carotenoid content of cow's milk is related to the size of the fat globules, and it appears that the carotenoids are associated with the membrane of the globules in both human and cow's milk. According to Kon & Mawson (1950), the vitamin A content is high at the beginning of lactation (100 i.u./g fat or more on the 3rd and 4th days). It falls rapidly to mean values of 46 i.u. in the 3rd week and 30 i.u. at the 20th, after which it may rise slightly. The carotenoids also decrease during the first 3 weeks, falling from about 20 μg/g fat at the outset to 4·3 in the 3rd week, but from the 4th week the value, around 3·6 μg/g fat, shows no definite trend. By means of a mathematical formula Kon & Mawson (1950) were able to correct the values of vitamin A of different samples to make them comparable despite the stage of lactation. In this way vitamin A and carotene content are found to increase with the age of the mother; this age effect is unrelated to parity and is so far unexplained. Consumption of the vitamin by the mother increases the content in the milk fat, but only for a few days. Carotenoids when eaten have little effect on the carotenoids and none on the vitamin A in the milk fat. β-Carotene constitutes only about one-fifth of the total carotenoids in milk and supplies some 3–4 % of the child's vitamin A intake. Kon & Mawson found that among the carotenoids, lycopene was more abundant than β-carotene, but it is not known to have any physiological significance.

Vitamin D is present in very small quantities in breast milk. Kon & Mawson (1950) found only 0·13–0·41 i.u./g fat, rather less than that reported by Drummond, Gray and Richardson (1939) and roughly the same as the amounts found by American workers (Harris & Bunker, 1939; Polskin, Kramer & Sobel, 1945). Assuming a fat content of 4 %, the daily intake of a 12 lb. baby taking 1 l. milk would be about 16 units. Supplements of vitamin D do not appear to alter the content of calcium or of phosphorus in breast milk (Macy, Hunscher, McCosh & Nims, 1930), or the content of the vitamin except in massive doses, but the mother needs adequate vitamin D for her own safeguard. When mother and child are unclothed the baby may receive direct irradiation and possibly he gets significant amounts of irradiated fats from the mother's areola and his own hand.

The vitamin B_1 in breast milk increases slowly in the first days of lactation, from 3 μg/110 ml. on the 3rd day to 9 on the 10th (Kon & Mawson, 1950). After the 5th week there is no consistent trend. The content responds slowly to the amount of the vitamin eaten but does not rise above 20–35 μg/100 ml. The riboflavin content, on the other hand, is altered in a matter of hours by the mother's food and has no maximum level. It increases during the first days of lactation, and Kon & Mawson (1950) found that riboflavin was present on average in a concentration of 26 μg/100 ml. in the milk of English women.

The concentration of vitamin C is probably higher in colostrum than in milk; otherwise it varies with the mother's intake, changing markedly in a matter of days. Commonly there are about 4 mg ascorbic acid in 100 ml. milk. The concentration cannot be raised above about 10 mg/100 ml., a value reached when the mother is taking 300 mg daily. The concentration falls slowly when the mother's intake is reduced and there is then a danger of the mother's being depleted, especially when she is feeding twins. The concentration in milk is above that of the serum; this difference may be due partly to the booster effect which the mammary gland is able to produce, just as free amino-acids are in higher concentration in milk than in serum. One may conjecture, however, that the effect may in part be produced by the ascorbic acid of colostrum bodies that may be seen in milk, for it is known that white corpuscles hold a much higher concentration than blood plasma and continue to do so when the plasma is depleted.

Minerals. Estimations or qualitative analyses have been made of many elements in breast milk. Of principal interest are calcium, phosphorus, sodium, potassium, chlorine and iron. The calcium and phosphorus of milk vary greatly in concentration in different women, although the level of secretion by one woman is relatively constant. Many workers have reported a range of calcium content between 19 and 40 mg/100 ml. This is in contrast to the constancy of plasma calcium, which ranges at most from 8·2 to 11·6 mg/100 ml. The phosphorus content of milk ranges between 10 and 20 mg/100 ml. (Kon & Mawson, 1950) giving a Ca : P ratio of 2·2 after the first weeks.

There is less sodium and potassium in human than in cow's milk, the range being from 11 to 19 mg/100 ml. for sodium and from 48 to 65 mg/100 ml. for potassium, approximately one-third of the concentration in cow's milk (Macy, 1949). The iron ranges from 0·09 to 0·2 mg/100 ml. (McCance & Widdowson, 1951) and the copper from 0·5 to 0·6 μg/ml. (see Kon & Mawson, 1950), the values in both instances being greater than for cow's milk. The many estimations of chloride made by Miller and his co-workers (Kermack & Miller, 1951) suggest that chlorides increase in concentration when the breast is lax; early in the day the concentration is less than later, and it is also less when the milk flow is abundant than when it is scanty. The range is from 30 to 150 mg/100 ml.

SUMMARY

The composition of breast milk depends primarily on the phase of lactation. Whereas the contents of protein, fat and sugar are virtually uninfluenced by diet, the concentration of the vitamins depends partly on what the mother eats.

Recent analyses of the amino-acids suggest that they are not present in significantly different proportions in human and cow's milk and that the reasons for the advantages of breast milk for a baby must be sought elsewhere.

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Artificial Infant Feeding

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It can be accepted as axiomatic at the present time that the basic food substance for the artificial feeding of healthy infants is cow's milk. In the first place, therefore, attention must be directed towards a comparison between human breast milk and cow's milk. Ideally, the substitute food should approximate to nature's food as closely and as exactly as possible. Towards the end of the last century this seemed to be a simple and relatively easy procedure and was the basis of what was called the percentage method of infant feeding. The protein, fat and carbohydrate content of the two milks was known and a little juggling with cow's milk, sugar and water provided the satisfactory modification. The advances of science and chemical analysis soon revealed differences, particularly in the proteins and fats of the two milks, and then the different amino-acid composition and the varying amounts and proportions of the many mineral substances began to render the problem increasingly difficult and complex. To-day, a perusal of current paediatric text-books and handbooks on the artificial feeding of healthy infants and a cursory glance at the spate of pamphlets, booklets and advertisements of the manufacturers of infant feeds leaves one disconcerted, overawed and aghast. Not unnaturally, the impression is being created, particularly in the minds of the uninitiated, that the whole subject is confused and chaotic. Indeed, the position might almost appear to be that, before any intelligent advice can be given on substitute or bottle-feeding, technical knowledge of the chemical composition of the innumerable constituents of breast and cow's milk must be profound, precise and exact. Surely this pseudo-scientific approach has been overdone, and has not the time come when we should return to a more simple, commonsense and realistic attitude?

It is submitted that fundamental principles seem to have been buried beneath a welter of secondary, minute or perhaps even unessential details. To-day in the streets of our cities and towns we can see countless happy, healthy and contented babies reared on artificial feeds by parents with a very wide range of intelligence, and it is difficult to feel that all have followed current professional advice, assuming that this has been sought. Let me reiterate that I am only concerned with the feeding of healthy infants; the problems in connexion with sick or premature infants will be dealt with by other speakers.

The principles to be observed are not really very complicated, and differ quantitatively but not qualitatively from those involved in meeting the nutritional requirements of any mammal, man or otherwise, with due allowance for the factors of growth and development. Human beings, for at least their first 6 months of life, are carnivora.

Nutritive values of cow's and human milk

In the first place, brief reference will be made to some of the known facts concerning the nutritive value of cow's milk in comparison to breast milk, as well as to some of the gaps in our knowledge. Such an approach is an essential pre-requisite for a proper appreciation of the problem of artificial feeding of all babies.

Protein. As a healthy infant has to double its birth weight in 6 months and treble it by its first birthday its protein intake must be of major importance. Cow's milk has more than twice the protein content of breast milk. During the first 6 months of life, infants fed on undiluted cow's milk show a steady increase in percentage of body nitrogen. This is in contradistinction to those breast-fed, who do not quite maintain their birth percentage of nitrogen (Stearns, 1939). Since, therefore, the body of the infant fed on cow's milk contains a greater percentage of nitrogen, the amount of intracellular fluid and salts must also be greater. The potassium retention in an artificially fed infant is considerably above that of a breast-fed infant and the same may be assumed to be true of sodium and phosphorus. Why this is so and what influence it may or may not have is not yet known; presumably it is in some way associated with the greater amount of protein in cow's milk and the different amino-acid pattern of the two milks. Williamson (1944) has compared breast milk with half-strength cow's milk and found that the former contained over three times as much cystine as the latter. There was less methionine in human milk, but no significant differences in the total sulphur-containing amino-acids. The stools of infants artificially fed when compared to those breast-fed have been shown (Ross, 1950) to contain very few amino-acids as determined by partition chromatography, and the chromatographic pattern was not constant. The higher protein content of cow's milk may, in part at least, explain these differences. Further investigations, however, on these lines (Ross, 1951) suggest that the protein of human milk is almost completely metabolized, and the absorption of food is so efficient that the faeces of the breast-fed baby contain only amino-acids provided by the intestinal flora and intestinal secretions. The significance of these findings has yet to be assessed.

Fat. The total fat content of the two milks is not dissimilar, but the acids of human milk differ from those of cow's milk in that they do not contain butyric or other lower acids. Indeed, human milk, in regard to its component acids, has more resemblance to a typical margarine-fat blend than to butterfat (Hilditch & Meara, 1944).

Minerals. There are significant differences in the mineral contents of the two milks, but these are subject to wide variations depending on the diet. On average, breast milk contains only about one-quarter the calcium of cow's milk, but there is a much greater percentage absorption of the former, probably due to the different bacterial content and hydrogen-ion concentration of the intestine of the breast-fed baby associated with the higher lactose content. The amount of iron present in human milk is about three times that of cow's milk and an iron-deficiency anaemia develops in artificially fed infants unless additional iron is prescribed.

Vitamins. In general, breast milk contains more vitamins A and C but less vitamin B_1 and riboflavin than cow's milk; vitamin D is inadequate in both (Kon & Mawson, 1950). Scurvy is very rare in breast-fed infants, probably because the diet of most nursing mothers contains adequate amounts of vitamin C. It would seem that breast-fed infants need only small amounts of vitamin B_1 in contradistinction to those that are bottle fed, but little is known concerning their riboflavin requirements.

Calories. Most of the calories that an infant expends are derived from the fats and carbohydrates, and there is no significant difference in the calorie values of human and cow's milk.

Nutritive needs of the human infant

Turning now from the theoretical and more scientific aspects to practical application, it is immediately apparent that the large number of artificial infant-foods available and in use demonstrates in no uncertain manner that these are a long way from being as satisfactory or ideal as human milk. In this connexion the first question that arises is what are the essential nutritive requirements of a healthy growing infant. It is platitudinous to state that the food intake must be adequate; without this, normal growth and development cannot proceed. There is broad agreement on the calorie intake required, namely 50–60 Cal./lb. body-weight/day. With regard to total fluid intake, here again most authorities agree that $2\frac{1}{2}$ oz./lb. body-weight/day may be accepted as the average. Thus, a feed containing 20 Cal./oz. and given in the proportion of $2\frac{1}{2}$ oz./lb. body-weight/day will satisfy the fluid and calorie requirements of most healthy babies. Controversy and difficulties, however, arise over the modifications of cow's milk that should be made to meet the above-mentioned criteria. It is universally recognized that whatever fluid is fed to the infant must be sterile, and in the first place, therefore, the cow's milk mixture must be free from all bacteria and micro-organisms. Dried or powdered milks have many practical advantages, and fresh cow's milk should never be used unless it has been boiled or pasteurized. Drying, boiling, pasteurizing or evaporating cow's milk have one common advantage in that they render the casein curd less dense and tough and therefore easier of digestion. In the interests of all concerned simplicity must be our motto, and powdered milks made from unmodified cow's milk have much to commend them; the required feeds are easy to prepare, satisfactory to the baby, very convenient for the mother and can readily be modified to suit the conveniences and idiosyncracies of the individual baby.

Modification of cow's milk for infant feeding

Protein. Two problems now arise on which professional opinion is far from unanimous. The questions are (*a*) what modifications are really necessary, and (*b*) in what quantities and at what intervals should the infant be given its feeds. Our paediatric teachers of a generation ago were rather apprehensive of the relatively high protein content of cow's milk and taught that this should be reduced to a level more approximate to that of human milk. 'Protein indigestion' as it was called is now considered a bogey, since we have learned to modify the protein curd before its ingestion. Indeed, in these days it is almost impossible to obtain protein curds in the stools of infants for demonstration to medical students. The paramount importance of an adequate protein intake in both health and disease is now universally recognized, and in this connexion tribute must be paid to the protracted and invaluable investigations and research of our colleagues in the nutritive and metabolic fields. In consequence of these advances in knowledge the high protein content of cow's milk is considered to be very far from a disadvantage, and almost all infants will tolerate, and benefit from, full-protein cow's milk.

Fat. With fat the matter is a little different for reasons already mentioned, hence the demand for half-cream milk mixtures. Fat intolerance is still a clinical entity, as the secretion of lipase in the infant is poor, and if produced the resulting disturbance can be extremely troublesome.

Carbohydrate. Attention has earlier been directed to the fact that an infant derives most of its calories from fat and carbohydrate. If, therefore, fat has to be reduced, a compensatory increase in the amount of carbohydrate is essential. As cow's milk contains less than two-thirds the carbohydrate in human milk, the necessity for additional carbohydrate is clear. Opinions differ on the best form of carbohydrate for this purpose, but personally I favour the readily available commodity, cane-sugar. I have yet to be convinced that glucose, or dextrose-maltose mixtures, which are more expensive and not so palatable, have any advantages.

Amount of feed and interval between feeds

Until quite recently the amounts in a feed and the intervals between feeds have been rather rigidly defined, but it is becoming more and more appreciated that an infant's desire for food as well as its need are subject to considerable variation from time to time and from day to day. Why should an infant, who through no fault of his own has to be bottle fed, be still further jeopardized by being deprived of satisfying his individual needs and desires like his breast-fed brother? I cannot subscribe to the opinion that 2½ oz. fluid/lb. body-weight/day is the optimum in all instances and will provide all that an infant needs, or that the quantity of each of the individual feeds should be obtained by dividing the total by the number of feeds. This is a ritual much too rigid and uncompromising and ignores the baby's appetite, preferences and desires. Such a procedure is against all the rules of nature. Amounts in a feed and intervals between feeds should be elastic and flexible, permitting the individual baby to exercise at least a little personal choice and selection. Do let us remember that babies are individuals. Wallgren (1945) has shown from a detailed study of nearly 400 normal breast-fed infants that there is no significant correlation between body-weight and milk consumption, and that the amount a particular baby may take is unpredictable. In Birmingham for the last year or more we have been using a modified self-regulating feeding schedule for artificially fed babies, with most pleasing results. The baby is offered a feed and allowed to suck until satisfied, but no attempts are made to force feeds after his requirements, as judged by disinclination to take more or passing off to sleep, have been met. Detailed charts showing amounts in a feed have been kept for some of these babies over a period of time. The results have been most instructive and fully confirm Wallgren's observations on breast-fed babies.

Though I am not in complete agreement with the views expressed by some that a healthy baby cannot be overfed, experience has taught that underfeeding is a very much more common fault than overfeeding.

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Difficulties of Infant Feeding

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There is no field of practical infant management of greater importance than that connected with the quick, precise and satisfactory solution of problems concerning alleged or real difficulties in infant feeding. In all spheres of paediatric practice there is a regular flow of such disorders, and each example needs individual consideration in order to find the correct solution. Like many other apparently simple clinical matters, the proper assessment of a feeding difficulty may call for the highest ability, either in detecting an unsuspected minor disturbing influence or in recognizing a potentially serious organic cause. A good understanding of the relationship between mother and infant is necessary as a background; an infant-welfare clinic will usually provide abundant training and experience in the social and psychological factors involved. Perhaps the increasing tendency for a mother to discover a feeding difficulty is a sign of present-day anxieties and, not least, is often a reflexion of sociomedical difficulties in accommodation, adaptation or adjustment, arising out of the housing problems of young parents.

Psychological considerations. Undoubtedly, worries and troubles in the mother's mind are the essential matter in very many instances; they may arise through the sheer lack of common sense of a young, ignorant and somewhat emotionally immature woman, or strangely enough, they may be the reaction of the elderly, knowledgeable mother who is carrying an unusual load of anxiety about her first and possibly last baby. A careful and quietly observant approach must be made by the doctor wishing to discover the whole truth. A mother may be quite reticent over the facts really helpful for understanding her problem as a whole. It is certainly no occasion for hasty and superficial methods. The whole story quietly told and explored is essential. An air of sympathetic understanding of the mother's feelings and sense of responsibility must be revealed, and a genuine wish to understand and help must be shown.

There is reason to believe that some cases of difficult feeding fail to reach a satisfactory solution because the doctor, midwife, nurse or health visitor will not attend to these simple preliminaries. A clear, quick and accurate analysis can only be made when the doctor is really informed upon the subject. The adviser should have a thoroughly competent knowledge of the physiology of lactation. A wrong decision, and one which may at the same time give the mother a false sense of security may simply deepen the difficulties.

It is here that mention should be made of the many very simple technical errors in breast feeding that can be easily adjusted, but that may unfortunately be quite overlooked, or worse still, grossly exaggerated, and may lead on to bad advice about possible weaning. Also, most regrettably, that pernicious story is still all too often heard about human milk not being suitable to the baby, a remark which might be regarded as the highest evidence of the lowest clinical ability in paediatrics. Doctors or midwives must remember their special responsibility in guiding the mother through the crucial first month of efficient, normal, natural lactation, which will give her effective confidence to continue and enable the baby to have a flying start in nutrition, and even, probably, provide certain psychological advantages.

Early feeding difficulties may be very disturbing to the lactational mechanism itself, and vicious cycles may quickly arise. There is much to be said, therefore, for a preventive approach, which concerns itself particularly with maintaining the association between mother and baby ('rooming-in') during the puerperium, so that an early, self-controlling procedure of nursing is created; sudden changes are thus avoided from hospital routine to home management. Otherwise many young and inexperienced mothers tend to worry themselves into infant-feeding difficulties and to develop a fatalistic attitude towards a failing lactation. Success in avoiding the difficulties breeds contentment with a real feeling of achievement and understanding in the mother, and it should be remembered that the achievement of success may mean well for subsequent occasions. On the other hand, unsolved and disappointing breast-feeding difficulties can run seriously counter to maternal hope and may occasionally give rise to some sense of inferiority.

The mother's health. Analysis of a feeding difficulty will obviously include such factors as the health of the mother and baby, including the presence of congenital defects or debilitating sepsis or prematurity. The mother's health is important. She may have a badly planned daily routine and is declining through excessive zeal and over-conscientious and obsessional attention to detail, through insufficient rest and sleep, or through a post-puerperal anaemia. In addition, she may have an anxious worried feeling about her husband, about finance, and about her domestic work, all leading her to imagine that the baby is unwell and to suspect that her own milk is unsuitable. Clearly a check on all such possibilities is only part of the individual clinical investigation. No detail in advice must be omitted. Distraction through excess of visitors, lack of peace and comfort at feeding times, coupled with apprehension through lack of experience and sound advice, may all be important. Many mothers contend heroically with most embarrassing circumstances, in noisy overcrowded flats with very poor facilities, and where their relations with their neighbours are spoilt perhaps by a restless, crying, ill-fed baby that chooses the night season for its major disturbances. Sometimes a noisy flat overhead will prevent a baby from following its own essential rhythm of sleep so that the nervous reaction creates a feeding difficulty. Lack of fresh air and long confinement through the winter in a top flat without access to the open air are bad factors contributing to the situation.

Such are some of the common, modern factors to be remedied when possible. Small doses of phenobarbitone may be extremely useful in some circumstances. Much of the disharmony in mother and baby can be prevented or diminished by reassurance and advice from an experienced mothercraft sister, an almoner, or the doctor if not too busy to appreciate the real significance of the small causes alleged. Infant-welfare centres can certainly excel in such work, and where antenatal, lactational training can be given too, an even greater defence against this kind of feeding difficulty can be built up. Experienced nurses are convinced that most difficulties in breast and bottle feeding arise from the mother's lack of knowledge and experience in looking after a new baby. The mother may be genuinely nervous of handling the baby and lack confidence in her ability to care for it properly, or she may not understand the importance of holding the baby comfortably or how to overcome its tendency to swallow air. Distension of the stomach with air may be a major factor in difficult feeding. It may arise in a variety of ways, but it should be recognized that the baby's own quick, restless temperament and method of approach to the meal are contributory. How much of the last is cause or effect cannot be elucidated without some understanding of the baby's early emotional responses, which should certainly not be neglected in studying infant feeding and its difficulties. In their varied responses babies are definitely individuals, a fact which emphasizes again the difficulties that overelaborate, mechanical routine in infant feeding may create. A self-demand programme, especially in breast feeding, has often remedied difficulty arising from a mother's too conscientiously clock-regulated routine. An intelligent mother soon learns how long it takes to feed her healthy, vigorous baby, and the breast is often emptied in a surprisingly short time. Dawdling about at breast feeding is unwise; it leads to exhaustion of mother and baby. Air may be swallowed during continued sucking at the emptied breast. Ten minutes at each breast is the aim. Difficulty will arise through the baby's fatigue if its efforts at the breast are unduly prolonged. It is better to preserve the baby's vigour by a complementary feed rather than allow such secondary upsets to occur. Peculiar disturbances of rhythm are occasionally seen; for instance a baby aged 6 weeks took 7 oz. at the 6.0 a.m. feed; at 10.0 a.m. he was sound asleep and showed no interest when put to the breast, but with persuasion he took 1 oz.; he then slept soundly till 4.0 p.m. when he woke and took a normal feed. Clearly there was no real difficulty in this instance.

Difficulties may arise if parents have busied themselves too much with book knowledge so that each minor detail of the child's health is overexaggerated. The mother should study her baby intelligently and without fear, and so learn by observation, if not by instinct, her own baby's requirements but, as a skilful ward sister put it, 'it is important that the baby should be really wanted in the home', otherwise the feeding difficulties appear to be endless. The psychological situation is, thus, continually important and often strikes the dominant note.

In breast feeding, inadequately developed nipples, or nipples recurrently sore or cracked, or over-engorged breasts which are painful to the mother and which make it almost impossible for the baby to take proper hold of the nipple constitute special difficulties but suggest their own remedies. An adverse psychological reaction or undue apprehension on the part of the mother may lead to failure of the preliminary mechanism that 'lets down' the milk within the gland so that the initial pumping action of the baby's mouth is less effective and difficulty quickly becomes apparent.

The feed. A complementary artificial feed may not be immediately acceptable. A change of taste or temperature or differences between the nipple and the teat may disconcert the infant. A rate of flow that is too rapid may frighten or overpower the baby, or one that is too slow may tire it and cause air to be swallowed. A change of the person holding the baby for a complementary or supplementary feed is not wise, for 'it's bad enough to have different food but it's the "last straw" if mother is not even holding him'.

Many mistakes of technique can be made in bottle feeding. The bottle may be held at the wrong angle to the mouth; the person feeding may be inattentive; the feed may be too hot or too cold, or badly mixed or inconsistently sweetened; the food or the person preparing it may be unnecessarily changed, causing the texture or taste to be unfamiliar; the milk if peptonized may have become bitter if treated for more than 20 min.; lactic-acid milk may have curdled through heating to about 100° F. All such mistakes are obviously matters of possible importance.

Causes of difficulty in both breast and bottle feeding may be a block in the nasal airway; a silent sore throat and pharynx or a cold leading to loss of appetite; overheating or chilling; irritation from a wet or rough napkin; gastric air-bolus; too great delay in feeding; or even the presence of the teat under the tongue when no amount of effort is in the least successful! However, after all these matters have been attended to, there is still the inexplicable factor that some persons can persuade most babies to take their feeds and some persons cannot; but continuous concentration, a steady pull on the nipple or the teat, a comfortable nurse and baby, with a warm pleasing feed, a peaceable atmosphere, together with optimism, all combine towards avoidance of difficulty in feeding.

Primary metabolic disorder is rarely concerned with difficulty in feeding. Occasionally a drug with an irritating taste may be secreted with the breast milk. Trials of so-called galactogogues such as thyroid extract may convey a diarrhoeal influence to the feeds. An untreated cretin, after several weeks of life, may have appreciable difficulty in maintaining its feeding status. Allergic sensitivity to cow's milk may be very real and call for special measures. Lethargy in neonatal jaundice may bring the added danger of dehydration unless a means is found of supplying enough fluid by frequent small feeds or by temporary oesophageal feeding. The newborn infant that suffers a big loss of weight in the first week of life becomes lethargic and loses appetite, so that a short period of oesophageal feeding may be necessary to restore vigour and weight. Refusal of food by a baby with a previously good record, may be the first sign of infective toxaemia and requires alertness to make a diagnosis and, if necessary, special measures to supply fluid and prevent aggravation of the condition by dehydration. Retropharyngeal abscess or even irritant pharyngitis may seriously interfere with normal swallowing. Anorexia in a baby with gastro-enteritis is a grave sign.

Mentally abnormal infants. Abnormal babies such as Mongols, and those likely to prove mentally abnormal through severe injury or asphyxia at birth, through cerebral agenesis, or through genetic causes, may show apathy towards feeding and reveal poor powers of sucking. Assessment of such conditions may be uncertain, but in severe cases with bad prognosis the feeding difficulty may not be pursued too officiously. Special care in diagnosis must be given to such possibilities as neonatal meningitis (*Bacterium coli*) or subdural haematoma.

Underfeeding. In the healthy baby underfeeding is the commonest problem. Difficulties arising from the feed itself are usually due to errors in the quantity or method of reconstitution rather than in the actual composition. A wide range in the efficiency of the intestinal digestive enzymes ensures that the healthy infant has a wide tolerance except for a gross excess of fat. In underfeeding or insufficiency from difficult feeding associated with air swallowing and secondary vomiting, the baby fails to thrive, constantly cries from hunger and abdominal pain, and has gaseous intestinal tension, constipation or a mucinous diarrhoea. The quantity of the feeds should be judged according to the infant's own metabolic requirements; some infants require up to 80 Cal./lb. body-weight daily to satisfy them and give a steady gain in weight, which frequently proves to be more than the standard. The average rate of weight gain is greatest in the first 6 months and greatest of all in the first 2–12 weeks.

Overfeeding. Mothers and some midwives frequently attribute certain signs, particularly loss of weight, to overfeeding, but this is rarely correct. Absurd miscalculations in making up feeds usually provide the answer. True overfeeding sometimes occurs in the breast-fed baby in the first 10 days, when the milk comes suddenly and in great abundance, and is likely to be of high fat content; the quantity and consistency of this creamy deep-yellow milk usually soon adjusts itself to the baby's needs and of course no question of weaning should ever arise in such circumstances.

Prematurity. Nearly all infants of less than 3½ lb. weight need oesophageal feeds for at least the first week of life. Feeding with a pipette or spoon is contra-indicated owing to the danger of the feed being inhaled. If the premature baby cannot suck from the ordinary boat-shaped bottle it needs oesophageal feeds, or it will become unnecessarily exhausted and fail to get enough, with dehydration and excessive weight loss as the consequences. The difficulties peculiar to the feeding of premature babies are many, but with good progress bottle or breast feeding can be introduced gradually.

Two-hourly feeding is contra-indicated; it causes too much disturbance and handling which in turn introduce their own difficulties.

Congenital malformation. Cleft lip and palate, micrognathia, choanal stenosis or atresia present particular problems. In the last, the feeding difficulty is a continual dilemma for the infant, but the difficulty can be slowly overcome by perseverance. Extreme degrees of micrognathia require a special technique in feeding to aid the mouth floor and bring the tongue forward so as to keep the pharynx accessible for swallowing. In severe cases of cleft lip and palate the infant's normal process of oral pumping and swallowing is quite disorganized, and oesophageal feeding may be necessary for a period, followed by spoon feeding, with the baby held as upright as possible; a shovel-shaped spoon makes the administration of fluid easier and reduces spilling. Actually, babies with cleft lip only, can usually pump better at the breast than at the bottle, since the breast tissue tends to close the gap. In cases, however, where an early operation is contemplated some surgeons prefer spoon feeding from the start, so that the baby becomes accustomed to the method of feeding which will be necessary after the operation. Congenital pyloric stenosis is still, unfortunately, too often confused with feeding difficulty, and the old-time story is still heard of changing the feed or even of taking the baby off the breast.

Malformations of the oesophagus may present some important problems in infant feeding. Mention must be made of the urgency of diagnosis in the swallowing difficulty caused by congenital oesophageal atresia; delay of more than 6 h in recognizing the condition is serious. Disorder caused by hiatus hernia may be alarming and the periodic, intractable vomiting, with possible haematemesis, which it causes may create acute feeding difficulty. Functional oesophageal regurgitation due to a lax cardiac sphincter (congenital chalasia) is a common cause of feeding difficulty, and much waste of food and failure to gain weight may occur; the tendency may continue for several months and may be remedied only by the ingenious use of thickened feeds or of small amounts given frequently, but spontaneous recovery is the rule. Simple rumination causes no disturbance in feeding.

Continuous gastric-drip feeding has particular use in the recurrent vomiting caused by lax oesophageal sphincter or sometimes in cases of hiatus hernia; at the end of 2 months if difficulty persists cereal feeding can be begun, the thicker medium tending markedly to diminish the oesophageal reflux. These techniques are very valuable for relieving this particular form of feeding problem. Spoon feeding also may have special temporary advantages. The feeding difficulties consequent upon gastro-enteritis, pyloric stenosis or any other organic alimentary obstruction will naturally be remedied only by treatment of the primary cause. Oesophagitis due to thrush infection may produce special difficulty in swallowing; treatment is urgent.

It is sometimes forgotten that difficulty in infant feeding may be due to underlying congenital heart disease; collapse or struggling or refusal to take the feed at any stage may arise from the cardiac embarrassment with or without cyanosis, and in one particular form where the coronary artery arises from the pulmonary artery, a kind of anginal reaction of exhaustion may be noticed during feeding.

When the infant swallows, a peculiarly specialized action with the tongue plays a highly important part in the pumping action and the pharyngeal projection of the fluid. In infancy, the tongue is naturally more anchored at the frenum than later, so that tongue tie, if it ever exists as such, is not a cause of feeding difficulty.

The Feeding of Premature Infants

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The general principles of infant feeding have to be modified for premature infants because of their physical handicaps and their greater nutritional requirements.

Handicaps

Premature babies have certain handicaps due to their incomplete development. Those affecting nutrition include weakness or absence of the sucking or swallowing reflexes, a tendency to inhale regurgitated fluids caused by absence of the coughing reflex, poor absorption of food and inadequate antenatal stores.

Management of handicaps. If the sucking and swallowing reflexes are completely absent, gavage (oesophageal feeding) is necessary, but weakness or rapid exhaustion of the reflexes may be overcome by giving small frequent feeds or divided feeds with 5 min rest during the feed.

The danger of regurgitation and inhalation may be reduced by delaying the commencement of feeding for several days, the smaller the infant, the longer the starvation period. When the infant is fed, it should be placed on its right side with the head raised; a clothed infant should be kept on the one or other side between feeds, when infants are nursed naked the head rolls over naturally on to the side; the feeds should be small and frequent to reduce distension of the stomach, and the smaller infants should be handled as little as possible, especially after feeding.

Poor absorption of food and poor antenatal storage are considered in the next section.

Nutritional requirements

The premature baby has greater nutritional requirements than the full-term infant, because of its relatively rapid postnatal growth combined with the handicap of inadequate storage.

The optimum rate of postnatal growth is not yet known. There is a tendency to aim at the maximum growth possible, but it is not yet proved that the maximum is the optimum. In the light of present knowledge it seems reasonable to use the intra-uterine rate of growth as a yardstick, and expect the gain in weight to average 5 oz. weekly for infants weighing less than 3½ lb. and from 7 to 8 oz. weekly for infants of greater weight (Streeter, 1920; Huggett, 1946; Crosse, 1949).

Such a rate of growth can be expected only after the initial loss of weight has been regained. Just as in the full-time child, an initial loss of from 6–10 % (1–1½ oz./lb.) of the birth weight may be expected, but the birth weight should be regained by about the end of the 2nd week, earlier in the larger babies and later in the smaller ones. If the intra-uterine rate of growth is then continued, a weight of from 6 to 8 lb. should be reached 2 weeks after the expected date of delivery, the weight being then equivalent to that of a full-time infant that has just regained its birth weight. The weight curve before the expected date of delivery lies below the intra-uterine weight curve, but should be parallel to it.

Calories

Before the age of 2 weeks the calorie requirement is very small because the basal metabolism is low and activity is relatively slight. After the first 2 weeks an allowance of from 55 to 60 Cal./lb. body-weight daily maintains a satisfactory rate of growth, provided that the calories are given in a suitable form and that the infant is cared for in a suitable environment.

Overfeeding, with its great dangers, must be avoided during the first 2 weeks of life. The requirements are small, and the danger of underfeeding is very slight, as is shown by the now common practice of starving premature infants for several days after birth. During these early days the premature baby, like the full-time one lives on body fat. It has relatively little of such fat but its small needs enable the fat to last for a long time.

It is important to know how the necessary calories should be provided. Certain requirements have been determined by balance tests and others by clinical trial, but there is still much to learn and no one can yet be dogmatic about requirements.

Protein

The smallest premature babies grow about twice as fast as full-time babies, and may require twice as much protein. It is, therefore, possible that human milk may not supply sufficient protein for the optimum growth of premature babies, even if allowance is made for their better utilization of protein, which varies inversely with the weight of the infant (Gordon & Levine, 1944).

Levine (1945) recommends a daily allowance of from 2·0 to 2·7 g protein/lb. body-weight for premature infants, and from 1·5 to 2·0 g for full-time ones; the full-time infant, however, does remarkably well on human milk although it obtains less than 1·0 g protein from a daily intake of 2½ oz./lb. body-weight.

At this point it may be well to remember the influence of artificial feeding on infant mortality. In a Birmingham survey of 1380 premature babies, feeding appeared to have little effect on the neonatal mortality, but between the ages of 1 and 6 months, the percentage mortality in breast-fed infants was 0·8 and in artificially fed infants 5·6.

Because of the low protein content of human milk various high-protein feeds have been tried, such as human milk with added protein and various modifications of cow's milk, and good weight gains have been reported (Langer, 1926; Hess & Lundeen, 1941; Magnusson, 1944; Gordon, Levine & McNamara, 1947). Feeding experiments in the Birmingham Premature Baby Unit support the view that high-protein feeds are well tolerated (Young, Poyner-Wall, Humphreys, Finch & Broadbent, 1950), but fail to show that they have any great advantage over unmodified human milk (experiments now in progress).

If it is necessary to substitute cow's milk for human milk, it is desirable to give a high-protein feed containing 3 % or more of protein. The extra protein ensures that the specific need for individual amino-acids will be met, the amino-acids being present in different proportions in human milk and cow's milk. There is no disadvantage in giving a relatively high percentage of protein provided that sufficient vitamin C is given also, to ensure complete metabolism of the amino-acids (Levine, Marples & Gordon, 1939; Dann, 1942).

Gordon *et al.* (1947) obtained the best results with a powdered, half-skimmed cow's milk mixture with added carbohydrate, and the Birmingham experiments confirm this finding. A cheap readily available mixture is 1 drachm of half-skimmed, dried milk to 1 oz. water, with the addition of 1 drachm sugar to every 3 oz. of the mixture; the percentage composition is protein 3·8, fat 2·1, carbohydrate 9·5, with 21 Cal./oz.

Fat

Determination of the respiratory quotient shows that premature babies tend to reject fat and utilize carbohydrate to a greater degree than full-time babies. The smallest babies usually have the least ability to absorb fat. The ability varies between individuals and even in the same individual at different times.

Many premature babies will tolerate high-fat feeds, but few utilize the fat well. If much fat is given, the excess is discarded in the stool and extra calories are required to compensate for the loss. There appear to be no advantages in high-fat feeding, and individual infants may even show intolerance to such feeding. On the other hand, provided an adequate dosage of fat-soluble vitamins is ensured, there are no dangers in giving relatively little fat, because fat is easily made from protein and carbohydrate.

It is a safe rule to assume that there will be difficulty in the utilization of fat during the first few months of life and to avoid high-fat feeds during that time. As the infant matures, the utilization of fat improves.

Carbohydrate

Premature infants utilize carbohydrates to a greater degree than full-time babies, and tolerate high proportions of non-fermentable sugars such as cane- or beet-sugar or dextri-maltose. High percentages of glucose or lactose may cause diarrhoea.

When cow's milk is the basis of the feed, a high percentage of sugar is necessary to compensate for the low percentage of fat and to allow the protein to be used for growth instead of being burnt for energy.

Fluid

Water accounts for a larger percentage of the weight at birth in premature than in full-time babies, from 80 to 85 instead of from 70 to 75.

Water-balance experiments have shown that the optimum daily fluid allowance after the 1st week of life lies between 2 and 3 oz./lb. body-weight. With less there is danger of dehydration, and if more is given the excess is eliminated as extra urine. Giving too little fluid is far more dangerous than giving too much, but it is necessary only to reach a daily amount of 2 oz./lb. body-weight by the end of the 1st week of life, and from 2½ to 3 oz. by the end of the 2nd week.

The volume of human milk that supplies the necessary calories supplies the necessary fluid also. If cow's-milk mixtures are used, they should not supply more than 24 Cal./oz. if the intake of a safe amount of fluid is to be ensured.

Mineral salts

Calcium and phosphorus. Premature birth deprives the infant of valuable antenatal-storage time and also increases the postnatal requirements.

Human milk has a low content of calcium and phosphorus; cow's milk has a higher content of both. An adequate supply of vitamin D is necessary for full utilization of the available calcium and phosphorus.

In the Birmingham Premature Baby Unit supplements of calcium and phosphorus are given to all premature babies from the 14th day as syrup of calcium lacto-phosphate, 7 minims/lb. body-weight daily. Radiographic examination of the wrists at the age of 3 months has been carried out on 227 infants thus treated, 109 breast fed and 118 fed on cow's milk. Bone changes suggestive of early rickets occurred in 13·8 % of the first group and in 25·4 % of the second. Clinical rickets was conspicuous by its absence in both groups.

Iron. The concentration of iron remains essentially the same throughout foetal growth, but the more rapid growth of the premature babies creates an extra demand.

Human milk contains little iron, and cow's milk still less, but there is no evidence that any useful purpose is served by giving extra iron before the age of from 4 to 6 weeks. After that age, administration is effective to prevent the development of an iron-deficiency anaemia.

Vitamins

The needs of the premature baby are greater than those of the full-time baby for the following reasons: the antenatal store is small, postnatal growth is more rapid, absorption of fat-soluble vitamins is poor, the need for vitamin C is increased with the use of high-protein feeds, and the need for vitamin D is increased by the extra needs for calcium and phosphorus. The following allowances are recommended:

Vitamin K, 10 mg daily until 2 days after the commencement of feeding.

Vitamin B complex, from the commencement of feeding; requirements unknown.

Vitamin C, from 50 to 100 mg daily from commencement of feeding, the larger dose being given to infants on high-protein feeding.

Vitamin D, from 3000 to 5000 i.u. daily from the end of the second week, or a massive dose commencing at the age of 2 weeks and repeated at monthly intervals.

Vitamin A, from the age of 2 weeks; requirements unknown.

SUMMARY

1. Gavage or bottle-feeding must be used to overcome early difficulties in sucking and swallowing.

2. Small frequent feeds may be necessary at first.

3. Full fluid and caloric requirements need be met only towards the end of the 2nd week of life.

4. From clinical experience, human milk appears to be a suitable food if given in sufficient quantity, 3 oz./lb. body-weight daily after the 14th day. Breast feeding tends to reduce the mortality rate of premature infants.

5. When cow's milk is used, as a substitute for human milk, a high-protein, low-fat, high-carbohydrate mixture is recommended.

6. Vitamins must be given in adequate amounts in a concentrated form.

7. Supplements of calcium and phosphorus are probably required after the age of 2 weeks, and of iron after the age of from 4 to 6 weeks.

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Skeletal Changes during Pregnancy and Lactation in the Rat: Effect of Different Levels of Dietary Calcium

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(*Received* 3 *February* 1951)

It is now well known that the skeleton serves as a store of calcium and phosphorus, and that a part of the mineral requirements of lactation is met by bone resorption. In an earlier communication (Warnock & Duckworth, 1944) the literature was surveyed and the role of the spongiosa as a source of minerals for lactation in the rat was studied under conditions of adequate calcium intake.

Certain interesting questions about the resorption of bone in lactation and its subsequent repair after lactation remain unanswered. In the first place, the extent to which the response of the skeleton to lactational needs is modified by differences in the level of calcium intake is unknown. Secondly, the extent to which cancellous and compact bone are individually resorbed in lactation and the degree to which these changes can be modified by alterations in calcium intake have not been studied. Thirdly, the extent to which calcium intake affects the capacity of cancellous bone and compact bone to recover after depletion does not seem to have been examined. Fourthly, it is not clear whether resorption of bone is the only process by which minerals are released from skeletal stores to meet the needs of lactation in the calcium-deficient animal. The possibility of demineralization of bone, that is of extraction of the mineral component without resorption of the organic matrix, must also be considered. Finally, it seems that no study has been made of changes in physical dimensions of bone in pregnant and lactating animals receiving diets differing in their calcium content.

The present experiment was designed to answer these questions and to afford an opportunity to study the effects of different dietary calcium levels on the reproductive performance of rats and their ability to rear their young. The diets were composed of natural feeding-stuffs, selected for their low calcium content. Most earlier investigators in this field used synthetic diets.

EXPERIMENTAL

Experimental design. A factorial design experiment was constructed to study the effects of calcium intake upon reproductive performance, skeletal change and organ size and simultaneously the effects associated with the gestation-lactation cycle. One hundred and forty-four female rats were randomized in twenty-four equal groups, of which six were assigned to each of four diets. Thus, in Tables 7–10, 12 and 13, each figure given under the heading 'Reproductive cycle' represents the mean of six individual values. The rats in the first group on each diet (designated 1 G) were killed at the end of the first gestation, those in the second group (1 L) were killed at the end of the first lactation. The animals of the third group (2G) completed a cycle of one gestation and the ensuing lactation, were mated again and killed at the second parturition. Those of the fourth group completed two reproductive cycles. Similarly, the 3G and 3L groups represented the third cycle. For present purposes the term 'reproductive cycle' is used to cover both the gestation phase and the ensuing lactation phase.

Diets. The basal diet and the composition of the four experimental diets prepared from it are given in Table 1. Blood meal (low-temperature dried) and dehydrated cod meal were used as sources of protein to keep the calcium content of the basal mixture low. The cod meal was prepared from cooked cod fillets carefully examined to ensure freedom from bone. The cod-liver oil contained 800 i.u. vitamin A and 100 i.u. vitamin D/g. Calcium carbonate was added at three different levels, with compensatory additions of starch to give constant totals of both. The diets were made up weekly.

Table 1. *Composition of basal diet and construction of diets A, B, C and D*

Basal diet	Parts	Basal diet	Parts
Ground wheat	23	Dehydrated cod meal	4
Ground oats	20	Dried brewer's yeast	3·5
Ground barley	20	Salt	0·5
Ground maize	20	Cod-liver oil	1
Blood meal*	6	Total	98

* W. and J. Dunlop Ltd., Dumfries.

Experimental diets*

Designation	Basal diet (g)	Starch (g)	$CaCO_3$ (g)	Ca in diet (%)
A	980	1·25	18·75	0·79
B	980	7·50	12·50	0·54
C	980	13·75	6·25	0·29
D	980	20·00	0·00	0·04

* All diets contained 18·7 % protein and 0·37 % phosphorus.

Animal management. Females were transferred to mating cages at 105 days of age. At the same time they were changed from the standard rearing ration of the colony (Thomson, 1936) to the appropriate experimental diet, which was fed *ad lib.* until the animals were killed. Young, active males were introduced to the mating cages at the rate of one to every two females. To avoid any effect of calcium deficiency on the fertility of the males they were only left in the mating cages overnight. During daytime they were kept separate and given the standard ration. No male was used in the same cage on two successive nights and all males were frequently given rests from breeding. Females were transferred from mating to individual cages about 5 days from parturition. On the 3rd day after parturition oversize litters were cut down to eight young.

The duration of lactation was 23 days and females not killed at the end of lactation were returned to the mating cages immediately.

Dissection. Animals were killed by coal-gas and dissected rapidly, major blood vessels being severed to aid in draining organs of blood. The surfaces of organs were dried, and weighing was carried out as quickly as possible. The right femur and tibia were freed from most of the adhering flesh, the fibula being discarded. The length of the femur was measured and the bone marked off into thirds. It was cut into three sections using a thin circular saw driven by an electric motor. The cuts were made at right angles to the surface of the bone at the point of section. The distal third of the femur was split into two fractions by separating the condyles from the metaphysis at the epiphyseal line. The four sections of the femur, in order, are referred to below as: the femur head, the femur shaft, the femur metaphysis and the femur condyles.

The tibia was divided into three parts in a similar manner. One cut was made at the point of junction of the tibia and fibula. A second cut was made half-way between the tibia-fibula junction and the proximal end of the tibia. The three sections of the tibia, in order, are referred to below as: the tibia head, the tibia shaft and the tibia distal section.

The different bone sections were freed as completely as possible from marrow by washing with a fine jet of water. Care was taken to avoid breaking off fine spicules of bone from the spongiosa. The portions of bone were then boiled in water and the remaining flesh was dissected off. Uniform cleaning of the medullary cavity close to the spongiosa is impossible unless the contents of the cavity are removed before boiling because of coagulation of the marrow.

Physical measurements. The cross-sectional areas of the proximal and distal surfaces of the femur shaft and tibia shaft of each animal were measured. *Camera lucida* tracings were made of the four surfaces recording the peripheries of the whole bone and of the medullary cavity. Area measurements of these were made using a planimeter. So that identical magnification would be secured on the various occasions when tracings of bones were taken, an accurately calibrated rectangular brass block, of size similar to the bone cross-sections, was used as a standard. Its image was projected on the tracing paper before each set of tracings, and the magnification adjusted. The term 'total cross-sectional area' is used below to include both the area of cross-section of the bone substance and the area of cross-section of the medullary cavity.

Chemical analysis. The portions of bone were extracted with boiling ethanol and dried to constant weight at 105°. Ashing to constant weight was carried out at 600°.

Presentation of results. Data for the physical measurements and chemical analyses of the various sections of the femur and tibia are tabulated by reproductive cycles and by diets. Organ weights are given in the same style. In each instance the mean values for gestation data and for lactation data are given under each reproductive cycle and for each diet. In addition, the means of all the gestation values and of all the lactation values, together with the mean of all results for each diet, are tabulated in the last three columns of Tables 7–10, 12 and 13.

When the animals were randomized among the twenty-four groups there were differences in mean group weight at 105 days of age. After the experimental data had been collected the effect of differences in initial weight at 105 days was calculated using analyses of covariance. Where the effect of such differences was significant, the regression coefficient indicating the effect and its standard error (S.E.) are given in the table. In these instances the means were adjusted for the differences in initial weight, and such mean group values are referred to as 'adjusted means'.

Below each section of Tables 7–10, 12 and 13 are given the standard errors of differences for the data in the section.

RESULTS

Reproductive performance. The mean weight increases of pregnant rats during gestation are given in Table 2. No significant difference in weight was found between rats receiving diets A, B and C during any gestation. The mean increase of weight in diet D rats was significantly less than the increases in diet A, B and C rats during the second gestation but not during the first or third. On each diet the mean increases were significantly greater during the first gestation than during the second and third, presumably as a consequence of continued maternal body growth.

Table 2. *Mean weight increases of pregnant rats during gestation*

Diet	First gestation (g)		Second gestation (g)		Third gestation (g)	
A	48·8	52·3	41·1	38·8	40·3	37·7
B	51·8		36·8		30·3	
C	56·4		38·5		42·5	
D	48·4		24·8		26·2	
S.E. of difference between mean of D and mean of A–C.	±4·40		±5·53		±7·08	

Table 3. *Mean weight losses of mother rats during lactation*

Diet	First lactation (g)	Second lactation (g)	Third lactation (g)
A	−18·2	−19·3*	−28·0
B	−13·0	−17·8*	−21·5
C	−10·3	−18·6*	−20·2
D	−18·8	−15·4*	−24·5
S.E. of differences	±6·75	±5·66	±13·04

* Corrected for number reared per litter.

Mean decreases in body-weight during lactation are given in Table 3. No significant difference was found between weight losses on the different diets for any lactation period. The weight changes were corrected for the number of survivors per litter. For the second lactation period there was a highly significant negative correlation between the number of survivors and the actual weight changes of the dams (regression coefficient $=3\cdot84 \pm 0\cdot95$), but not for the first and third lactations.

The weights of litters at birth and numbers of animals per litter are given in Table 4.

The birth weights of first litters were higher than those of subsequent litters, irrespective of diet. The difference arose from the greater number of rats in the first litters. A highly significant negative correlation was found between mean weights of newborn rats and the number of rats in the first litter, but not in the second and third litters. A correction was accordingly made for the first litters but not for the second and third litters.

Table 4. *Mean numbers and weights of rats in litters born on different diets*

Litter	Diet	Mean weight of litter (g)	Mean number in litter	Mean weight per rat (g)
First	A	61·05	10·89	5·75
	B	61·69	10·69	5·83
	C	58·71	10·25	5·83
	D	60·69	10·86	5·67
	S.E. of differences	±4·06	±0·77	±0·126
Second	A	47·23	7·61	6·25
	B	46·38	7·63	6·17
	C	51·54	8·41	6·20
	D	42·12	7·00	5·93
	S.E. of differences	±4·88	±0·81	±0·178
Third	A	49·79	8·27	6·11
	B	43·84	7·33	6·01
	C	44·41	7·00	6·48
	D	34·42	6·00	5·81
	S.E. of differences	±7·46	±1·23	±0·835

The lowest litter birth-weights occurred in litters from animals on diet D at the second and third parturitions. These lower values arose from a combination of lower individual weights and lower numbers in D litters. Although the average number of pups born was least in diet D groups during the second and third cycles, the reduction was not statistically significant.

A further analysis was carried out to test whether the reproductive performances, as indicated by the numbers per litter at birth and the birth weights of litters, were correlated between the three cycles. Animals with poor reproductive performance in the first cycle tended to perform badly in later cycles, but the correlations just failed ($P=0·06$) to reach the 5% level of statistical significance. No dietary effect was found in any of these analyses.

The mean weaning weights of rats of different litters from the four dietary groups are given in Table 5. The regression coefficients for the correlation of weight of weanlings and number of animals per litter are also given. At all stages there were highly significant negative correlations between the number of rats in the litter and the average weight of the rats weaned, and the values in the table have been adjusted for these correlations.

There were highly significant reductions in mean weaning weights of diet D rats for all reproductive cycles as compared with corresponding animals from A, B and C diet-groups. The average weaning weights of diet D rats were about the same for all three litters. However, for litters from rats on diets A, B, and C the average weight of weanlings was higher in the second and third litters than in the first. These values can be compared with the mean for the stock colony of the Institute during the period of this study, which was 46·09 g.

Table 5. *Mean weaning weights of rats from different litters*

(All values corrected for the number of rats weaned per litter)

Diet	First litter (g)	Second litter (g)	Third litter (g)
A	40·7	47·2	45·3
B	43·1	49·0	44·3
C	43·9	49·1	55·0
D	36·6	34·2	36·7
S.E. of differences	± 1·24	± 2·09	± 4·02
Regression coefficient: weight/number	− 3·61 ± 0·115	− 2·88 ± 0·355	− 2·02 ± 0·612

In the first reproductive cycle the average weight of diet A weanlings was less than the values for B and C weanlings by an amount that is barely significant. In the third litters the average weight of diet C weanlings was just significantly more than the values for A and B weanlings.

The data for dams completing three reproductive cycles were analysed separately. There was no evidence of mothers producing consistently heavy or consistently light weanlings.

The number of survivors per litter and numbers of litters successfully reared are given in Table 6.

Table 6. *Numbers of rats surviving to weaning together with the number of litters reared in each subgroup*

Diet	First litter*	Second litter*	Third litter*	Total*	Percentage survival
A	189/193 (25)†	99/104 (15)†	31/38 (5)†	319/335 (45)†	95·2
B	223/228 (30)	101/108 (17)	23/27 (4)	347/363 (51)	95·6
C	193/207 (27)	115/128 (18)	26/31 (5)	334/366 (50)	91·3
D	200/206 (27)	92/101 (16)	29/30 (6)	321/337 (49)	95·3
Total	805/834 (109)	407/441 (66)	109/126 (20)	1321/1401 (195)	—
Percentage survival	96·5	92·3	86·5	—	—

* First value gives the total number of survivors at weaning on the 23rd day; the second value gives the total number of young present on the 3rd day after oversize litters had been reduced to eight; value in parentheses gives the number of litters reared.

† Full complement of litters in first-litter subgroups = 30; in second-litter subgroups = 18; in third-litter subgroups = 6. Values less than these occur because some litters were born dead, some were killed by their dams and some were neglected.

In twenty-one out of 216 litters the young were either born dead, or the mother failed to rear them. The distribution of failures bore no relation to the diet of the dams.

The percentage of young surviving to the end of lactation fell in successive litters, but there was no significant difference between the dietary groups in the number of young reared.

Physical measurements of bones. The results from measurements of total cross-sectional areas of bones are given in Table 7. Medullary cross-sectional areas, expressed as percentages of the corresponding total cross-sectional areas are tabulated in Table 8. Data for the lengths of the femur and tibia are not given. The average increase in these lengths during the study was about 3% and no effect due to the level of dietary calcium was found.

Table 7. *Influence of level of dietary calcium on dimensions of the total cross-sectional areas of the femur and tibia shafts of rats during gestation and lactation*

(All values given in sq.mm. Adjusted means)

	Reproductive cycle*						Mean		
	First		Second		Third				
Diet	1 G*	1 L*	2 G*	2 L*	3 G*	3 L*	All G's*	All L's*	All cycles*
Proximal end of femur shaft									
A	8·40	8·19	9·03	9·01	8·95	8·50	8·79	8·57	8·68
B	8·49	8·47	8·53	9·11	8·85	8·87	8·62	8·82	8·72
C	8·50	8·36	8·75	9·28	9·27	8·99	8·84	8·88	8·86
D	8·14	7·72	9·10	9·09	9·98	9·29	9·07	8·70	8·88
Regression coefficient=0·013±0·0020									
S.E.†=0·305 (6); 0·176 (18); 0·124 (36)									
Distal end of femur shaft									
A	8·26	8·07	8·24	8·56	8·59	8·06	8·36	8·23	8·30
B	8·59	8·38	8·09	8·60	7·74	8·27	8·14	8·42	8·28
C	8·36	8·28	8·45	8·64	8·73	8·47	8·51	8·46	8·48
D	7·85	7·34	8·39	8·59	9·63	8·80	8·62	8·24	8·43
Regression coefficient=0·0150±0·0021									
S.E.†=0·332 (6); 0·191 (18); 0·135 (36)									
Proximal end of tibia shaft									
A	6·65	6·20	6·29	6·49	6·21	5·76	6·38	6·15	6·26
B	6·55	6·27	6·16	6·43	6·07	5·95	6·26	6·22	6·24
C	6·48	6·22	6·52	6·52	6·41	6·04	6·47	6·26	6·36
D	6·05	5·23	6·52	6·80	6·96	6·62	6·51	6·22	6·36
Regression coefficient=0·010±0·00195									
S.E.†=0·307 (6); 0·177 (18); 0·125 (36)									
Distal end of tibia shaft									
A	4·06	3·92	4·24	4·75	4·39	4·15	4·23	4·27	4·25
B	4·15	4·00	4·17	4·32	4·10	4·18	4·14	4·17	4·16
C	4·09	4·01	4·36	4·31	4·42	4·20	4·29	4·17	4·23
D	3·93	3·93	4·23	4·41	4·86	4·43	4·34	4·26	4·30
Regression coefficient=0·0078±0·00034									
S.E.†=0·171 (6); 0·099 (18); 0·070 (36)									

* See pp. 236, 237.

† Figure in parentheses gives the numbers of observations in the means to which the standard error refers.

The total cross-sectional areas of proximal and distal ends of the femur and tibia shafts showed no consistent evidence of continued peripheral growth during the experiment for animals receiving diets A, B and C, although there was an indication of an increase in size between the first and second cycles. The trend of diet D rats was significantly different from this.

During the first reproductive cycle the total cross-sectional areas of the proximal and distal surfaces of the femur and of the proximal surfaces of the tibia of rats on diet D were significantly less than the corresponding areas for rats on diet A, B or C. For the distal end of the tibia the total cross-sectional area in rats on diet D was the lowest, but not significantly less than the area for the corresponding diet A rats. In the third reproductive cycle the situation was reversed at all four surfaces. The mean values for total cross-sectional areas at all four zones during this cycle were significantly greater in rats on diet D than the largest value found in rats receiving diet A, B or C.

Table 8. *Influence of level of dietary calcium on the medullary cross-sectional areas, expressed as percentages of the total cross-sectional areas of the femur and tibia shafts, of rats during gestation and lactation*

	Reproductive cycle*						Mean		
	First		Second		Third				
	1 G*	1 L*	2 G*	2 L*	3 G*	3 L*	All G's*	All L's*	All cycles*
Diet	Proximal end of femur shaft								
A	22·8	31·7	27·6	34·3	31·8	36·7	27·4	34·2	30·8
B	24·4	35·5	30·1	38·2	29·3	36·1	27·9	36·6	32·3
C	26·2	40·9	31·6	39·8	35·5	41·6	31·1	40·8	35·9
D	26·8	51·9	39·8	57·9	46·6	52·9	37·7	54·3	46·0
	S.E. of differences† ±2·90 (6); ±1·67 (18); ±1·18 (36)								
	Distal end of femur shaft								
A	32·2	37·2	35·4	37·4	35·6	40·6	34·4	38·4	36·4
B	33·5	40·1	37·8	42·4	34·0	40·6	35·1	41·0	38·1
C	33·3	46·8	37·5	43·8	39·4	45·6	36·7	45·4	41·1
D	33·9	49·3	46·2	59·7	53·2	60·8	44·4	56·6	50·5
	S.E. of differences† ±2·38 (6); ±1·38 (18); ±0·97 (36)								
	Proximal end of tibia shaft								
A	25·3	28·4	24·3	27·5	24·4	27·3	24·7	27·8	26·2
B	24·7	31·5	24·6	30·1	23·2	27·5	24·2	29·7	26·9
C	26·2	34·0	26·9	29·0	25·8	29·7	26·3	30·9	28·6
D	27·6	42·4	35·8	48·5	37·0	45·6	33·5	45·5	39·5
	S.E. of differences† ±2·34 (6); ±1·35 (18); ±0·95 (36)								
	Distal end of tibia shaft								
A	12·9	17·6	14·7	19·8	14·3	20·9	14·0	19·4	16·7
B	13·6	20·3	16·0	22·7	15·9	20·9	15·2	21·3	18·2
C	13·4	28·3	15·3	23·8	19·0	25·4	15·9	25·8	20·9
D	16·1	33·8	30·6	47·9	35·8	48·6	27·5	43·4	35·5
	S.E. of differences† ±2·99 (6), ±1·72 (18), ±1·22 (36)								

* See pp. 236, 237.

† Figure in parentheses gives the number of observations in the means to which the standard error refers.

The total cross-sectional areas showed no evidence of cyclical changes with gestation and lactation.

When the area of the medullary cavity is expressed as a percentage of the total area of cross-section, increased values represent endosteal erosion. These percentages have been used since the total area of cross-section did not vary much in relation to the variation in the medullary cross-sectional area. Further, the use of percentages allowed individual variations in total bone size to be eliminated thus giving slightly more accurate comparisons. (Statistical analyses were in fact also carried out on the area measurements of the medullary cavity, but the results are not included here since they bring out no new feature.) In what follows, reference made to the percentage measurement might equally refer to the area of cross-section of the medullary cavity.

Comparison of the means of all cycles showed that the percentage medullary cross-sectional area increased as the level of calcium in the diet was reduced. This was true for all four zones. However, in no instance was the difference between groups receiving diets A and B significant. The values for rats on diet C were consistently greater than values for rats on either diet A or diet B with one exception—the difference between groups B and C just failed to reach the 5% level of significance for the proximal end of the tibia shaft. The medullary cross-sectional areas, as a percentage of total areas, of rats fed on diet D were strikingly greater than the values at all four bone surfaces of rats fed on diet C.

The data in Table 8 show the cyclical nature of endosteal erosion during lactation, and repair during the subsequent gestation. These changes occurred with all diets and at all shaft surfaces examined and became progressively greater as the level of calcium intake was reduced. In all instances the cyclical variations of rats on diet D were significantly greater than those of rats receiving other diets, but in no instance could differentiations be made between the variations of groups receiving diets A, B and C. Individual comparisons on means of adjacent subgroups sometimes failed to reach the accepted level of statistical significance, probably as a consequence of the relatively small size of the subgroups.

Cycles of erosion during lactation and repair during subsequent gestation occurred with all diets and at all shaft surfaces examined. When rats received diet A, B or C there was alternating resorption of bone from the surface of the medullary cavity during lactation and new growth of bone at that site during the ensuing gestation. These changes were reflected in the cross-sectional measurements of the medullary cavity. When diet D was given, an additional factor was involved. Here, because of continued periosteal growth, the recovery phase was characterized by both endosteal and periosteal increases in bone substance. To a minor degree there was some periosteal growth in rats receiving diet A, B or C up to the end of the second lactation, but only in rats on diet D was there marked periosteal growth at the same time as endosteal growth.

The areas of cross-section of bone substance are not given, but can be calculated from the data in Tables 7 and 8. The values showed cycles of erosion and repair in most measurements. Bone substance showed reduction in size at the end of lactation for all diets. The mean values for bone substance, when the figures for gestations and lactations were combined, showed progressively smaller cross-sectional areas as the level of calcium in the diet was reduced.

Chemical analyses of bones. Data for ash weight and ash weight expressed as a percentage of dry fat-free bone portions are given in Tables 9 and 12, respectively. Weights

Table 9. *Influence of level of dietary calcium, pregnancy and lactation on the weight of ash (mg) in sections of the femur and tibia of rats*

(Adjusted means)

	Reproductive cycle*						Mean		
	First		Second		Third				
Diet	1 G*	1 L*	2 G*	2 L*	3 G*	3 L*	All G's*	All L's*	All cycles*
Femur head									
A	121·7	90·3	124·6	107·3	130·9	94·8	125·7	97·5	111·6
B	109·9	89·0	115·0	107·8	122·4	111·1	115·8	102·6	109·2
C	120·2	82·0	122·6	100·2	124·8	99·9	122·5	94·0	108·2
D	101·0	52·9	76·8	41·7	83·8	41·0	87·2	45·2	66·2
Regression coefficient = 0·232 ± 0·0462									
S.E.† = 6·3 (6); 3·63 (18); 2·57 (36)									
Femur shaft									
A	65·4	57·8	69·3	66·7	75·8	61·5	70·2	62·0	66·1
B	64·7	60·6	66·0	69·0	73·0	67·5	67·9	65·7	66·8
C	63·0	52·6	67·7	59·8	71·2	60·9	67·3	57·8	62·6
D	61·3	32·8	45·9	25·2	51·8	27·1	53·0	28·4	40·7
Regression coefficient = 0·135 ± 0·0291									
S.E.†4·67 (6); 2·70 (18); 1·91 (36)									
Femur metaphysis									
A	69·0	43·6	65·3	4·78	66·0	45·1	66·8	45·5	56·2
B	64·8	45·1	56·6	4·59	59·6	48·6	60·3	46·5	52·7
C	70·7	37·1	58·6	4·26	57·6	44·1	62·3	41·3	51·8
D	51·5	27·6	35·2	1·86	40·3	16·2	42·3	20·8	31·6
Regression coefficient = 0·116 ± 0·0284									
S.E.† = 4·35 (6); 2·51(18); 1·78 (36)									
Femur condyles									
A	47·9	35·1	49·9	43·0	54·8	41·8	50·9	40·0	45·4
B	45·7	34·9	50·1	45·2	50·6	46·7	48·8	42·3	45·6
C	47·5	32·9	50·9	42·2	54·9	40·3	51·1	38·5	44·8
D	39·2	22·0	31·9	17·5	37·9	14·6	36·3	18·0	27·2
Regression coefficient = 0·099 ± 0·0198									
S.E.† = 3·04 (6); 1·76 (18); 1·24 (36)									
Tibia head									
A	110·0	73·8	102·8	84·0	108·6	80·5	107·1	79·4	93·2
B	104·6	76·9	92·6	80·9	102·6	89·4	99·9	82·4	91·2
C	104·0	65·3	101·2	78·1	101·6	78·6	102·3	74·0	88·2
D	79·6	39·0	57·0	29·8	68·1	32·2	68·2	33·7	51·0
Regression coefficient = 0·180 ± 0·0406									
S.E.† = 6·45 (6); 3·72 (18); 2·63 (36)									
Tibia shaft									
A	54·0	49·4	58·6	60·4	62·7	53·7	58·4	54·5	56·4
B	50·1	48·5	55·8	56·0	61·0	56·5	55·6	53·7	54·6
C	54·4	44·1	57·4	52·0	61·1	52·9	57·6	49·7	53·6
D	49·3	30·7	39·9	24·8	45·8	26·3	45·0	27·3	36·2
Regression coefficient = 0·123 ± 0·0219									
S.E.† = 3·52 (6); 2·03 (18); 1·44 (36)									
Tibia distal section									
A	61·3	58·8	65·0	64·9	68·8	59·7	65·0	61·1	63·0
B	62·8	58·7	63·5	61·9	64·0	63·8	63·4	61·5	62·4
C	64·7	56·0	63·4	61·2	68·6	62·5	65·6	59·9	62·8
D	59·6	39·3	43·2	27·8	47·5	28·2	50·1	31·8	41·0
Regression coefficient = 0·088 ± 0·0252									
S.E.† = 3·97 (6); 2·29 (18); 1·62 (36)									

* See pp. 236, 237.

† Figure in parentheses gives the number of observations in the means to which the standard error refers.

of dry fat-free bone have been omitted. Their fluctuations did not present any additional feature to warrant special consideration.

Lactation caused a reduction in the quantity of ash (and bone) and was followed by new bone formation during the next pregnancy. In varying degrees, an erosion phase followed by a growth phase was found in every section of the femur and tibia and on all diets. In only three instances was erosion not observed: small increases in ash weight occurred in the tibia-shaft sections of rats on diet A and on diet B during the second lactation and also in the femur-shaft sections of rats on diet B during the same lactation.

Table 10. *Influence of level of dietary calcium, pregnancy and lactation on the ash weight (mg) of the combined femur and tibia of rats*

(Adjusted means)

	Reproductive cycle*						Mean		
	First		Second		Third				
Diet	1 G*	1 L*	2 G*	2 L*	3 G*	3 L*	All G's*	All L's*	All cycles*
A	528	408	541	473	567	437	545	439	492
B	501	416	495	469	532	485	510	457	483
C	523	369	522	433	541	438	529	413	471
D	440	264	329	186	374	166	381	206	293

Regression coefficient = 1·0 ± 0·21

S.E.† = 30·5 (6); 17·6 (18); 12·4 (36)

* See pp. 236, 237.

† Figure in parentheses gives the number of observations in the means to which the standard error refers.

The magnitude of the depletion was not the same during all three reproductive cycles. In general, the quantity of ash (and bone) resorbed during the second cycle was less than that during the first and third. This smaller degree of resorption during the second cycle was seen in all sections of the femur and tibia.

Highly significant differences between rats on diet C and diet D were found in the ash weights of all bone sections. In comparisons between rats on diet B and diet C significant differences were found during the lactation phase, but not during the gestation phase. No uniform discrimination was possible between the corresponding means for ash weights from animals on diets A and B.

The combined ash weights of all sections of the femur and tibia are given in Table 10. Rats fed on diet A, B or C all showed gains in the weight of bone ash during the course of the experiment. The weight of bone ash of rats receiving diet D fell during the same period. A consideration of the summary of all reproductive cycles shows highly significant differentiation between values for ash weight in gestation and lactation. But a comparison between the ash weights of the different dietary groups allows for discrimination only between diets A, B and C combined and diet D. The one exception is a significant difference amongst the 'all lactation' values—the mean ash weight of bones from diet B rats was significantly greater than that of the bones from diet C rats.

The slight increase in bone length, referred to above, was reflected in the general increase in ash weights (and bone substance) of all portions except the distal portion of the tibia. In place of the overall increase in bone size of rats receiving diet A, B or C a decrease occurred in the quantity of bone in animals on diet D. This decrease appeared to be complete by the end of the second lactation, and the values obtained for ash (and bone) in the different portions at the end of the third lactation were not markedly different from those previously found.

Table 11. *Absolute and relative losses of ash during lactation from different parts of the femur and tibia of rats; mean values for three lactations*

		Diets A, B and C Ash lost		Diet D Ash lost	
Bone	Bone section	Weight (mg)	(%)	Weight (mg)	(%)
Femur	Head	23·3	19·2	42·0	48·2
	Shaft	6·6	9·7	24·6	46·4
	Metaphysis	18·7	29·6	21·5	50·8
	Condyles	10·0	19·9	18·3	50·4
Tibia	Head	24·5	23·8	34·5	50·6
	Shaft	4·6	8·0	17·7	39·3
	Distal section	3·8	5·9	18·3	36·5

The combined femur and tibia ash showed the following losses in mg during the first, second and third lactation, respectively: diet A 120, 68, 130; diet B 85, 26, 47; diet C 154, 89, 103; diet D 176, 143, 208. In all three reproductive cycles, drafts from rats receiving diets A and C were similar, but losses from both these groups were greater than from rats receiving diet B. However, this difference failed to reach statistical significance.

The absolute and relative losses of ash from the various bone portions during lactation are given in Table 11. Combined average values for diets A, B and C are compared with values for diet D. Under all dietary conditions the distal portion of the tibia showed the least evidence of resorption during lactation. The shaft portions also showed only slight resorption, except when the level of dietary calcium was very low. Then the femur shaft in particular showed a loss of ash approaching that of the femur head and metaphysis—portions that consistently lost the greatest proportion of ash during lactation.

A distinct cycle was observed in the percentage of ash in all portions of bone from animals receiving diet D. Lactation was always accompanied by a decrease in the percentage of ash, and pregnancy by an increase. Bone sections from animals fed diet A, B or C did not uniformly exhibit the same cyclical changes, although the indication of a cycle could be discerned in most cases.

Means of all gestation and lactation values for the percentage of ash differed significantly in all portions of bone from rats receiving diet D. Significant differences were not uniformly observed in animals receiving other diets. When the corresponding values for animals receiving diet A, B or C are combined, significant depressions of

Table 12. *Influence of level of dietary calcium, pregnancy and lactation on the percentage of ash in sections of the femur and tibia of rats*

(Regression coefficients not significant)

	Reproductive cycle*						Mean		
	First		Second		Third				
Diet	1 G*	1 L*	2 G*	2 L*	3 G*	3 L*	All G's*	All L's*	All cycles*
				Femur head					
A	71·3	68·3	70·5	71·5	71·2	68·3	71·0	69·4	70·2
B	70·8	67·4	70·4	69·2	71·1	69·1	70·8	68·6	69·7
C	70·7	67·2	70·2	68·6	70·4	70·1	70·4	68·6	69·5
D	69·8	62·3	65·0	54·7	64·6	50·0	66·5	55·7	61·1
			S.E.† = 1·73 (6); 1·00 (18); 0·71 (36)						
				Femur shaft					
A	75·3	75·1	75·3	75·4	75·6	74·8	75·4	75·1	75·2
B	75·3	75·4	75·7	76·2	75·6	75·4	75·5	75·7	75·6
C	75·2	74·9	75·5	75·5	75·3	75·6	75·3	75·3	75·3
D	75·5	72·8	73·4	71·0	72·6	69·5	73·8	71·1	72·5
			S.E.† = 0·77 (6); 0·44 (18); 0·32 (36)						
				Femur metaphysis					
A	70·0	68·5	70·5	65·8	69·6	67·8	70·0	67·4	68·7
B	70·2	68·1	69·8	68·7	69·7	68·2	69·9	68·3	69·1
C	70·0	67·0	68·8	67·6	68·3	67·0	69·0	67·2	68·1
D	68·4	61·6	64·9	56·0	64·3	50·5	65·8	56·1	61·0
			S.E.† = 1·44 (6); 0·83 (18); 0·59 (36)						
				Femur condyles					
A	63·3	61·2	65·1	62·6	66·4	62·8	64·9	63·3	63·6
B	64·4	61·0	63·9	62·4	65·7	63·2	64·6	62·2	62·3
C	64·3	59·2	64·2	62·4	65·2	62·6	64·6	61·4	63·0
D	63·0	53·5	59·0	45·6	59·6	42·5	60·5	47·2	53·8
			S.E.† = 1·54 (6); 0·89 (18); 0·63 (36)						
				Tibia head					
A	68·3	66·8	67·9	65·6	68·6	66·2	68·3	66·2	67·2
B	67·8	65·5	67·5	65·8	68·8	66·8	68·0	66·0	67·0
C	67·6	64·4	67·4	66·0	68·0	67·2	67·6	65·9	66·8
D	64·8	58·6	62·2	51·8	62·6	51·7	63·2	54·0	58·6
			S.E.† = 1·57 (6); 0·91 (18); 0·64 (36)						
				Tibia shaft					
A	74·1	74·5	74·5	74·8	73·9	73·5	74·2	74·3	74·2
B	73·0	74·9	74·2	74·7	74·4	73·9	73·9	74·5	74·2
C	74·0	74·3	74·0	74·3	74·2	74·9	74·1	74·5	74·3
D	74·5	73·1	72·6	70·5	71·6	69·2	72·9	70·9	71·9
			S.E.† = 0·59 (6); 0·34 (18); 0·24 (36)						
				Tibia distal section					
A	70·7	70·2	70·8	68·7	71·1	70·4	70·9	69·8	70·3
B	70·7	69·6	70·7	70·6	71·1	70·5	70·8	70·2	70·6
C	70·1	67·8	70·1	68·7	70·7	71·3	70·3	69·3	69·8
D	69·7	68·6	69·6	65·0	68·4	65·4	69·2	66·3	67·7
			S.E.† = 0·96 (6); 0·55 (18); 0·39 (36)						

* See pp. 236, 237.

† Figure in parentheses gives the number of observations in the means to which the standard error refers.

percentage ash in lactation are found for the femur head, metaphysis and condyles and for the tibia head and distal section.

In rats on diet D, percentage-ash values tended to decrease progressively. This tendency was most marked in those portions of bone that exhibited the greatest degree of depletion—head, metaphysis and condyles of the femur and the head of the tibia.

Organ weights. Adjusted mean values for weight of spleens and ovaries and unadjusted mean values for weight of thyroids are given in Table 13. There was no evidence of continued growth of liver during the experiment and no cycle during pregnancy and lactation. Kidneys continued to grow throughout the experiment from a mean value of 2·07 g during the first reproductive cycle to 2·32 g during the third. The increase was accounted for by continued growth of the body. There was no cycle in kidney size during pregnancy and lactation. The adrenals showed a significant decrease of about 7% during the experiment, but pregnancy and lactation had no specific effect on size.

Table 13. *Influence of level of dietary calcium, pregnancy and lactation on the mean weights of spleens, ovaries and thyroids of rats*

	Reproductive cycle*						Mean		
	First		Second		Third				
Diet	1 G*	1 L*	2 G*	2 L*	3 G*	3 L*	All G's*	All L's*	All cycles*
	Spleen (adjusted weight in g). Adjusted means								
A	1·05	0·88	1·08	0·89	0·99	1·01	1·04	0·93	0·98
B	1·00	0·94	0·99	1·06	1·09	0·96	1·03	0·99	1·01
C	1·36	0·90	1·20	0·92	1·11	1·00	1·22	0·94	1·08
D	1·23	0·91	1·17	0·86	1·31	0·93	1·24	0·90	1·07
	Regression coefficient=0·00218±0·00071 S.E.†=0·114 (6); 0·066 (18); 0·046 (36)								
	Ovaries (adjusted weight in mg). Adjusted means								
A	93·3	50·3	96·1	61·1	100·4	68·6	96·7	60·0	78·3
B	85·0	56·6	99·4	64·3	100·0	78·5	94·8	66·5	80·7
C	83·2	55·2	95·5	58·4	107·2	76·1	95·4	63·2	79·2
D	103·9	57·3	100·0	59·1	97·8	65·6	100·5	60·6	80·6
	Regression coefficient=0·154±0·053 S.E.†=8·56 (6); 4·94 (18); 3·49 (36)								
	Thyroid (weight in mg)								
A	24·1	26·0	41·2	36·5	43·1	39·9	36·1	34·1	35·1
B	21·5	32·3	38·7	41·7	41·0	45·8	33·8	39·9	36·8
C	23·4	25·1	35·7	38·8	39·0	42·2	32·7	35·4	34·0
D	25·3	32·3	30·3	32·1	32·7	31·5	29·4	32·0	30·7
	Regression coefficient not significant S.E.†=4·96 (6); 2·86 (18); 2·02 (36)								

* See pp. 236, 237.

† Figure in parentheses gives the number of observations in the means to which the standard error refers.

For the spleen there was a highly significant difference between the mean values for all gestations and all lactations. The gestation values of animals receiving diets C and D were about 20% higher than those of animals receiving diets A and B. At the end of lactation the effect of diet on spleen weight had disappeared.

All groups showed a cycle in ovary weight during each reproductive cycle. There was a steady increase in the mean weights of ovaries throughout the experiment in animals receiving diet A, B or C. In the rats on diet D there was a larger increase during the first pregnancy and thereafter the mean size was maintained, though the difference between D and the other diets is not fully significant.

There was continued growth of the thyroid gland throughout the experiment on all diets. The increases were greater than could be accounted for by increases in body-weight in rats receiving diets A, B and C. In rats on diet D the growth was less than that on the other diets.

DISCUSSION

The above results show that bone resorption during lactation and bone replacement during the next gestation were found in all regions of the femur and tibia irrespective of the level of dietary calcium. Different parts of these bones contributed widely different quantities of mineral during the resorption phase. The zones rich in cancellous bone—the head and metaphysis section of the femur and the head of the tibia—were the prime sources of mineral when the dietary provision of calcium was near to adequacy. The quantity of mineral contributed by the condyles of the femur was less, although the proportion eroded was similar.

The portions mainly composed of compact bone—the shaft of the femur and the shaft and distal portion of the tibia—were less subject to resorption. They contributed but little mineral, either absolutely or relatively, unless the diet was very inadequate in calcium. When this happened, the compact bone was attacked and the proportion resorbed was almost as great as in the more labile cancellous zones. The distal portion of the tibia remained at all times less subject to resorption than other regions of compact bone.

If the shaft portions are taken to represent true bone, contaminated with but minimal amounts of marrow and with relatively few vascular channels to retain non-osseous material, there was no evidence of selective solution of mineral from the organic matrix of bone in this experiment. Should a partial demineralization of the organic matrix take place during the resorptive changes of lactation, cycles would be seen in the percentages of ash in the shaft sections. This was not found. No cyclical variation in the percentage of ash in shafts occurred in rats eating diet A, B or C, though in diet D rats there were slight but significant cyclical changes. Even though the leg bones of the diet D animals were eroded to the extent of losing almost half their mineral content, the percentage of ash in the shaft portions fell only by 3–5 %. It is even doubtful whether this small reduction in the percentage of ash was real; microscopic examination of the bones of rats on diet D showed the small cavities, which characterize porotic bone, filled with organic debris.

In the zones that are rich in cancellous bone the cycles in the percentage of ash are probably spurious. It is not likely that demineralization occurred in that portion of bone constituting the spongiosa yet failed to occur in compact bone. Those bone portions containing large amounts of spongiosa had only a small proportion of the non-osseous organic matter in the interstices removed by our cleaning method. In resorption of spongiosa the weight of true bone decreases but the amount of non-osseous organic material remains unaltered, or may even increase as this material takes the place of resorbed bone spicules. Only a portion of this non-osseous organic material is removed during ethanol extraction, the remainder appearing in analysis indistinguishable from bone organic matrix.

This view on bone resorption in lactation agrees with the histological observations that when bone resorption is provoked by dosing with parathyroid hormone the organic matrix and bone salt disappear simultaneously without preliminary demineralization or even the formation of osteoid borders (McLean & Bloom, 1941). Further evidence supporting a view of general uniformity of composition of uncontaminated bone was given by Baker, Butterworth & Langley (1946) who studied the composition of true bone cleaned by microdissection. They found no difference in calcium and nitrogen content of femur cortex and femur cancellous bone, or between dead (sequestra) and living bone. Only between the cancellous bone of infant ribs and the cancellous bone of adult ribs did they find a slight but significant difference in ash content: 62·7 % for the infant and 65·8 % for the adult.

The level of calcium in the diet was without effect on the total cross-sectional areas of the femur and tibia, except with the diet of lowest calcium content. When the diet contained only 0·04% calcium, these external dimensions of the bones were smaller than those of rats fed on the other diets in the first reproductive cycle. During the course of the experiment the bones of rats fed on diet D gradually increased in size until, by the end of the third lactation, they were equal to, or larger than, the bones of the rats fed on the higher calcium diets. It appears that the capacity for external, or periosteal, bone growth is not impaired by calcium deficiency, even when this dietary deficiency is intensified by periods of calcium loss in lactation.

Selection of the points at which to cut the leg bones to expose surfaces for measurement was made so that these surfaces differed in their proximity to cancellous bone. It was originally thought that there might be a greater degree of erosion of compact bone in regions close to actively resorbing masses of spongiosa. This was not so as far as the surfaces we selected to expose were concerned. Erosion took place during lactation at all medullary surfaces, and no particular zone gave a better indication of the effect of the different diets.

An opportunity is presented by these results to compare physical methods of bone examination with chemical methods for the purpose of discriminating between diets in their capacity to minimize drafts on the skeleton. Both methods of measurement allow discrimination between the effects of diet D and all other diets. However, such discrimination is of little value since diet D is patently inadequate in calcium. In the comparison of diets B and C both the chemical method and the physical method, when the area of cross-section of the medullary cavity is expressed as a percentage of the total area of cross-section of the bone, are equally satisfactory. On the other hand, neither method discriminates consistently between the effects of diets A and B.

Cox & Imboden (1936) concluded that 0·49 % calcium in the diet of rats was the most suitable level for sustaining repeated pregnancies and lactations in the mother and normal weaning weight and ash content in the young. Diet B of the present experiment had a similar calcium content (0·54%), and the lactation drafts on the skeleton were significantly less when this diet was given than when diet C was fed. However, the combined femur- and tibia-ash weights were not significantly lower for diet C rats than for diet B rats. The effect of the lower calcium intake on bone is thus restricted to greater losses in lactation with correspondingly greater new bone growth after lactation. It is interesting to note that increasing the calcium content of the diet by 50 %, as in diet A, or decreasing it by 50%, as in diet C, was without an effect beyond this slight increase in resorption and repair in diet C rats.

These changes found for leg bones probably reflect the changes in the skeleton as a whole. In the present study the diet D rats lost 40% of the combined femur and tibia ash during the first lactation. During the subsequent gestation 37% of the loss was made good by new leg-bone growth. Comparable data for the whole skeleton of rats given diet D show that 32 % of the total skeletal ash was lost during the first lactation and that 40 % of the loss was regained during a subsequent period of 3 weeks (unpublished data).

Although diet D was markedly deficient in calcium, as shown by the state of the bones, the shortage had little effect on reproduction and mothering instinct. The fertility of the dams was unaltered and the survival of their young was unaffected. Their capacity to produce litters of normal size was but slightly impaired and their tendency to gain less in weight during pregnancy was, perhaps, a reflexion of this. The smaller weaning weights of litters from females eating diet D were probably the outcome of calcium deficiency reducing milk production. Nevertheless, complete failure of lactation was no more common in these females than in others with more plentiful supplies of calcium, even under the severe strain of repeated pregnancies and lactations.

In the experience of some other groups of workers, breeding and nursing performance is impaired by calcium deficiency. Boelter & Greenberg (1943) found that, when adult female rats were transferred to a diet severely deficient in calcium (0·01 %), only half of them produced litters, in spite of repeated matings, and that the majority of the litters was either still-born or the young died shortly after birth. To secure a repetition of pregnancy to the stage of producing two litters was almost impossible. However, they found that females transferred to their deficient diet when adult, but not earlier, had normal oestrous cycles, that six to eight pups were usually born in each litter and that the average weight of the pups was normal.

Bodansky & Duff (1941*a*, *b*), using the diets of Cox & Imboden (1936), reported that, even when the calcium content of the diet was as low as 0·017 % (phosphorus content 0·245 %), there was little effect on the size or weight of litters. But they found, as did Cox & Imboden, that the fertility of the females fell to about 50 %.

The divergence of our results from those of other workers is mainly in connexion with the fertility of the dams and the viability of the newborn. The principal difference between our experiments and those mentioned above is that our diet was composed of natural feeding-stuffs whereas theirs were synthetic. In addition, our male rats received a diet adequate in calcium. This raises the question of whether other inadequacies may have characterized the synthetic diets. In fact, Boelter & Greenberg

(1943) emphasize that their deficient diet, when supplemented with calcium, yielded inferior results to that of their normal stock diet. Simmonds (1924), using a natural diet, although with casein as the main source of protein and containing more calcium (0·103%), secured good reproduction but some interference with lactation, as judged by the growth of litters and the general weakening of the dams.

It seems probable that an uncomplicated deficiency of calcium in the diet of the rat has only a small effect on reproductive processes and a moderate effect on lactation. In other words, the avidity of the depleted maternal skeleton for calcium, in the face of an inadequate dietary supply, does not impair reproduction, but the rate at which the maternal skeleton can yield calcium to remedy a severe shortage in lactation is below that necessary to sustain a full flow of milk. It is to be emphasized that these results were obtained with females reared on an adequate diet from birth to the beginning of their first pregnancy. Also it is doubtful whether comparable results would be obtained in species where the young are more mature at birth than rat pups. The calcium content of the rat litter is low, the foetal skeletons not competing seriously with the maternal skeleton for calcium. In other species—for example sheep—the reverse is true: the ewe can lose bone substance during gestation on a low-calcium diet while the foetus develops an almost normal skeleton. Where the foetus is a serious competitor for meagre calcium supplies a detrimental effect on the reproductive performance of the dam might be expected.

The reason for enlargement of the spleen during gestation in rats receiving diets C and D is not clear. It was the only change in an organ that allowed discrimination between the effects of diets B and C. The condition was not a reflexion of the degree of calcium deficiency, for the enlargement was of the same order in both diet C and D rats.

Neither the stage of the reproductive cycle nor the level of dietary calcium affected other organ weights, except those of the ovaries. Here cyclical changes of the same order as those reported by Perlman & Leonard (1947) were found. The greater increase in ovary weight during the first reproductive cycle in calcium-deficient rats is of obscure significance.

SUMMARY

1. Bone growth and bone erosion in rats fed on diets of different calcium content during gestation and lactation have been studied.

2. During lactation erosion occurred irrespective of the level of calcium intake.

3. Erosion was confined principally to regions rich in cancellous bone, but resorption of the shaft bone at the surface of the medullary cavity was found in all regions.

4. There was no evidence of selective solution of bone mineral independent of erosion.

5. In a comparison between chemical and physical methods of bone examination, certain physical measurements provided as accurate a means for discriminating between the effect of diets of different calcium content as chemical methods.

6. The weights of liver, kidneys, adrenals and thyroid were not affected by differences in the calcium content of diets. At the end of the first gestation ovaries from severely calcium-deficient rats were considerably heavier than ovaries from adequately

fed rats. The spleens of rats with lower calcium intakes showed a weight gain during gestation which disappeared during the subsequent lactation.

7. Fertility of the dam and mothering instinct were not impaired, even in severe calcium deficiency. The weight of the young at birth and the number of young in a litter were not affected by the level of calcium intake by the dam. Weaning weights of young from severely deficient dams were lower.

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A Balance Sheet of the Estimation of Energy Intake and Energy Expenditure as Measured by Indirect Calorimetry, Using the Kofranyi-Michaelis Calorimeter

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(Received 30 July 1951)

In a recent review, an expert committee of the Food and Agricultural Organization of the United Nations (1950) sets out existing physiological knowledge of energy requirements from food in relation to practical rationing problems. Throughout their report the committee again and again draw attention to the gaps in present knowledge and to the need for further investigations. One method of assessing food requirements is the measurement of energy expended (in terms of heat loss) during various physical activities. Direct measurements of the heat loss of man under varying conditions have been made. Such methods of direct calorimetry possess the advantage of great accuracy, but human calorimeters are expensive and require great skill in use. Though they are invaluable as instruments for determining basic physiological principles, they can be of little help in tackling problems of energy requirements under conditions of everyday life. Indirect calorimetry (the assessment of energy expenditure from measurements of rates of oxygen consumption) is a more practical possibility but, even by these means, the drawing up of an energy balance-sheet presents great difficulties.

The metabolic cost of numerous activities has been accurately determined by measurement and analysis of samples of expired air collected in Douglas bags. The size and clumsiness of these bags limit their range of use in everyday life; their small capacity in relation to lung-ventilation rates limits the time for which one bag can be used. For these reasons estimations of the cost of complicated activities, such as take place in modern industry, are always difficult and time consuming, so much so as to be often wellnigh impossible. Many attempts to draw up a daily cost of energy expenditure by persons of different occupations have been made; these have been reviewed and summarized by Orr & Leitch (1937–8). Such estimates, though probably essentially correct, depend of necessity on much guess-work and have a wide range of error.

The development by Kofranyi & Michaelis (1940) of a compact, light, portable calorimeter is a great technical advance. This instrument is essentially a dry gas-meter, weighing only 4 kg and easily carried on the subject's back; it measures directly the volume of the expired air, and at the same time collects a continuous sample of a small constant fraction, which is available for subsequent analysis. The calorimeter has already been used in Germany for a big survey of energy expenditure in industry (Lehmann, Müller & Spitzer, 1949–50), and for a detailed study of the metabolic cost of the housewife's manifold activities (Droese, Kofranyi, Kraut & Wildeman, 1949–50). Similar studies have not been made in industry in this country.

In this laboratory Orsini & Passmore (1951) have compared results of the cost of a range of simple standardized work, using both Douglas bags and the Kofranyi-Michaelis calorimeter and found a good agreement between the two. But before undertaking a survey of the energy cost of various activities in British industries, we thought fit to test the instrument and the reliability of methods of indirect calorimetry in a laboratory study. The plan was that the subjects of the experiments should live in the laboratory for a period, during which a complete record of activities would be made, and the cost of sample activities determined by means of the calorimeter. In this way estimates of total energy expenditure could be obtained. At the same time accurate measurements of food intake would be made, and an assessment of the total calories available for expenditure provided. If a reasonable agreement were found between these two estimates, this would provide a measure of the reliability of the methods, and might provide a basis of confidence for results obtained later in industry under conditions less suitable for prolonged observations.

METHODS

General. Five students, men between 19 and 25 years, acted as subjects and lived in the Department for 2 weeks. During 13 consecutive days, a detailed time record was kept of all their activities, all food consumed was weighed and the metabolic cost of about thirty separate activities determined for each subject. The plan was to spend the first 3 days in sedentary life within doors, then to have 5 days of hard physical work, and finally a second period of 5 days of sedentary life. The two sedentary periods passed according to plan. The subjects spent the whole time in light activities and undertook no hard work. Except on two or three occasions when they went on small errands, they did not leave the building.

For the hard physical activity, outdoor track-walking was chosen. This is hard work, a steady and continuous activity, whose metabolic cost can easily be determined. The movements involved are those to which the subjects were of necessity accustomed in their everyday life. Unfortunately, the weather proved unkind, and wind and rain made conditions variable and often unfriendly on the grass track 1030 yd. around the University athletic ground. On one afternoon conditions were so bad that climbing up and down stairs in the laboratory was substituted for walking. Unfortunately three of the subjects developed blisters and minor orthopaedic trouble which on occasions restricted activity. Further, John, one of the fittest, developed mumps in the middle of the experiment and was only available for heavy work on 3 days, during which he walked 50 miles. Evan, whose feet prevented his exercising on one day, walked 52 miles. Iain, George and Alistair walked 66, 71 and 76 miles respectively, spread out over the 5-day period. The speed of walking varied between 4·2 and 4·8 m.p.h. In addition to the walking, a dance was held at the end of the work period. The metabolic cost of the various dances was determined without difficulty. One subject, George, played two games of Rugby football. The direct cost of these games could not be measured, but an estimate was made by comparison with other activities.

Choice of diet and estimation of its energy value. Because of uncertainties about the accuracy of estimates from food tables it was decided that the energy value of the diets should be obtained by chemical analysis of duplicate samples. To allow each subject to choose the whole of his diet quantitatively and qualitatively was impractical because of the very large number of analyses which such a procedure would have involved. Thus, to reduce the number as far as possible, about half of the total energy value of the diet each day was provided as a basic diet which was exactly the same for each subject. For the remainder of the diet each man was allowed to eat as much as he liked of bread, butter, sugar, bramble jelly, processed cheese and milk. Beer or cider was available at the evening meal. All these, as well as a sample of the basic diet for each day, were analysed separately. The energy values were calculated from the chemical analysis for moisture, protein, fat and ash. Carbohydrate was obtained 'by difference' and the energy value estimated from the carbohydrate, fat and protein content using the Atwater factors, 4, 9, 4.

The eating habits of the men had been observed during a preliminary study, and the pattern of the meals during the 13 experimental days was as close to this as possible.

The selection of dishes was in agreement with the likes and dislikes of the subjects. The pattern of the daily diet was as follows:

Breakfast: porridge or cereal and fruit juice; cooked dish.
Lunch: meat, fish, cheese or egg dish; potato and other cooked vegetable; baked or steamed pudding; biscuits.

Tea: cake or biscuit.

Dinner: meat, fish, cheese or egg dish; potato and other cooked vegetable or salad; hot or cold dessert, e.g. trifle, fruit pie.

At each meal weighed quantities of bread, butter, cheese, sugar, bramble jelly and milk were placed before each subject so that he could eat as much as he desired of any of these six foods. The amount eaten by each was ascertained by weighing what was left at the end of the meal. All foods were placed in covered vessels to avoid loss of weight by evaporation. Cellophane bags were found to be ideal for the bread. Either coffee or tea was served with each meal. No allowance was made for the energy value in either tea or coffee.

Using this technique it was possible to present a varied menu each day and also to limit the number of chemical analyses to about twenty samples for the whole period. Care was taken to avoid the use of foods difficult to sample, e.g. very fat meat or bacon, and for the same reason, frying as a method of cooking was avoided. Composite dishes, such as fruit pies, meat cooked with vegetables as in stews, trifles and salads, were prepared by weighing each ingredient for each subject and also for the sample for analysis. Where cooked, these were weighed and served in individual Pyrex dishes. This technique proved to be acceptable to the subjects, and, as none left any of the basic diet on his plate, was equally successful from our point of view.

One subject, John, did not consume the basic diet for 5 days while he was suffering from mumps. Weight records of each item eaten were kept and, during that period, the estimate of the energy value of his diet was made from food tables. While ill he consumed mostly milk, sugar, eggs, fruit juices, bread and butter. The energy value of these particular foods is fairly definite and the error introduced from this method of calculation should be very small.

Indirect calorimetry. Basal metabolic rates, and rates lying at rest, were determined with a Benedict-Roth spirometer, assuming a respiratory quotient of 0·78. All other rates were obtained using the Kofranyi-Michaelis calorimeter. Gas analyses were carried out in duplicate using the Haldane apparatus. All estimates covered periods of at least 6 and at most 15 min, the average being 10 min. As conditions varied owing to the weather on the outdoor track, estimations on each subject were made each morning and afternoon when walking was undertaken. Speeds varied slightly in the different periods. For each subject the range of cost for sitting activities was small, and for light indoor activities not great. Accordingly, selected values have been chosen for sitting activities and for light indoor activities. These have been used throughout. To split up these two groups into their separate activities would have involved an immense labour and, in the conditions of our experiment, would probably add little to the accuracy.

Record of activities. The problem of recording, from minute to minute and from day to day for 13 days, the multifarious activities of five active young men seemed, on first consideration, to demand the assistance of five observers. Under other conditions, with less intelligent or less enthusiastically co-operative subjects this is probably so. In the present investigation, however, each subject acted as his own observer, timing and recording his activities in great detail on a specially designed activity chart. Each chart, foolscap size, covered 8 h of the day, providing a 3 in. square for each hour. The hour squares were subdivided into sixty smaller squares for recording by minutes and a small compartment for notes on the activities engaged in during the hour. The minute squares were quickly filled in with red or blue pencil, a change of colour indicating a change of activity. Code letters of the various activities indicated the meaning of each change of colour from blue to red or red to blue. These code letters were explained in a legend at the bottom of the chart, but it was possible for the subject to record any doubtful or undefined activity by an additional code letter, the meaning of which was then added to the legend. Two 8 h charts recorded the details of each waking day and from them it was possible to calculate the total time spent daily on each of the various activities recorded. To guard against possible forgetfulness or inaccuracy when out of the Department, each subject had a notebook in which details and time were noted for later transcription to the chart.

RESULTS

Metabolic cost of various activities. Table 1 shows the results of all the estimations of metabolic cost during the 13 days, except those obtained on John when febrile, which are shown separately in Table 2. Tables 3–7 record, for each subject for the total period of 13 days, the total time spent in each classified activity, the estimated cost of the activity and the total calories expended. The activities are arranged according to the total calorie expenditure over the period. The justification for grouping all sitting activities together and all light indoor activities together has already been explained. The total period spent in bed each night, from the time of lying down until rising in the morning, was costed at the basal metabolic rate on the assumption that any period when the metabolic rate was above the basal rate would approximately balance the period during sleep when calorie expenditure might be expected to fall a little below the basal rate. Any other period during the day which was spent lying down was recorded under the heading ‘lying at rest’.

‘Indoor walking’ was assumed, from careful observation, to be carried out at approximately 3 m.p.h., except by George, who generally moved somewhat faster than the others.

‘Stair climbing’ covers the routine daily movements about the building carried out as nearly as possible at 100 stairs/min whether climbing or descending. ‘Stair exercise’, on the other hand, covers a period of carefully timed and controlled exercise which replaced ‘track walking’ on a particularly inclement afternoon. The apparently large amount of time spent in climbing stairs is due to the subjects having to live and sleep at the top and feed on the ground floor of a building not provided with a passenger lift.

Energy balance of the subjects. Table 8 shows the energy value of the food consumed and Table 9 the final balance for each subject. For John, George and Evan, who each gained a little weight up to 0·34 kg, the estimated energy value of the food absorbed is up to 2100 Cal. above the estimated expenditure. This represents an average daily difference of less than 160 Cal. or up to 5 % of the total. Alistair was estimated to have used 5000 Cal. less, and Iain 3100 Cal. more, than he ate. But for Alistair, against the excess of food, there is the 2·66 kg gain of weight; against Iain's food deficit a loss of 2·49 kg of body-weight can be set. Any attempt to convert these weight changes into corresponding calories could only be the wildest guess in the absence of precise measurements of changes in body-water content.

Table 1. *Metabolic cost of various activities (in Cal./min)*

Activity		John (67 in., 131 lb.)	George (68 in., 152 lb.)	Alistair (72 in., 166 lb.)	Evan (72¾ in., 175 lb.)	Iain (75 in., 183 lb
Basal		1·12, 1·13, 1·11	1·20, 1·15, 1·26	1·14, 1·09, 1·21	1·31, 1·16, 1·27	1·22, 1·27, 1·2
Lying at ease		1·4, 1·4, 1·2, 1·4	1·4, 1·6, 1·4	1·4, 1·6, 1·5	1·5, 1·6, 1·6	1·5, 1·4, 1·3
Sitting:						
At ease		1·5	1·9	1·8	1·9	2·0
Calculating		1·5, 1·2, 1·7	2·3	2·0	2·0	2·1
Reading		—	2·2	1·9	—	—
Writing a letter		—	1·9	2·2	2·0	2·2
Listening to the Boat Race		—	—	—	2·0	2·5
Playing Canasta		2·0	2·1	2·1	1·9	2·5
Unspecified (selected value)		1·8	2·2	2·1	2·0	2·3
Standing at ease		1·5	2·2, 2·1	2·0, 2·3	2·5, 2·4	1·8, 1·9
Light indoor activities:						
Peeling potatoes		—	2·7	—	—	—
Washing dishes		—	—	3·7	3·4	3·9
Washing socks		—	4·1	—	—	—
Cleaning shoes		—	—	—	3·3	4·7
Laboratory work		2·5	3·5	3·9	3·9	3·3
Dressing, washing, shaving		2·8	4·1	3·6, 3·7	4·5, 4·1	3·9, 4·2
Making beds		—	—	7·6	—	—
Unspecified (selected value)		2·7	4·0	3·8	4·0	4·0
Walking:						
Indoors	2·4 m.p.h.	4·3	4·7	3·8	—	5·1
	3·0	—	5·6	5·5	5·3, 5·4	5·1, 5·9
Outdoors	4·0	—	8·2	—	—	—
	4·2	8·2	9·0	—	10·4, 11·3	9·8
	4·4	9·1	10·3, 9·5, 10·1	9·7, 9·8, 9·9	10·7	—
	4·6	10·1	—	10·6	—	11·0, 11·2
	4·8	10·2	10·6	12·0	11·6	11·2, 13·7
Up and down stairs	97 per min	6·6	8·0, 8·9	9·6, 10·1	9·9, 9·5	9·6, 9·3, 9·0
	116	8·5	8·4	10·3	10·4	11·8
Dancing:						
Foxtrot		—	—	—	5·2, 5·2	5·8
Waltz		—	—	5·7	—	7·1
Rumba		—	7·0	—	—	—
Petronella		—	4·7	—	—	—
Eightsome reel		—	7·5	—	8·1	7·5

Table 2. *Energy expenditure of John, lying in bed febrile*

Temperature (° F)	Cal./min
99·6	1·31
101·0	1·34, 1·44
101·6	1·54

During the first 3 days of sedentary life, all the subjects consumed more than they expended. The excess appetite was no doubt partly psychological and could be

Table 3. *Energy expenditure of John over 13 days*

Activity	Time		Energy expenditure	
	h	min	Cal./min	Total Cal.
Track walking	11	8	9·2	6,170
In bed	56	14	1·11	3,750
Light sedentary activities	27	0	1·8	2,920
Sitting at ease	28	38	1·5	2,580
Walking (about 3 m.p.h.)	4	52	5·0	1,460
Light standing activities	6	21	2·7	1,030
Stair exercises	1	54	8·5	970
Dressing, shaving	3	59	2·8	670
Stair climbing	1	1	7·0	430
Lying at rest	1	37	1·4	140
Sundry activities	1	55	—	180
Total	6 days			20,300
Sitting up in bed	69	57	1·6	6,720
Lying in bed (febrile)	44	29	1·35	3,600
Lying in bed (afebrile)	41	58	1·11	2,800
Lying in bed (afebrile) (low fever)	11	28	1·25	860
Out of bed	—	8	4·0	30
Total	7 days			14,010
Grand total	13 days			34,310

Table 4. *Energy expenditure of George over 13 days*

Activity	Time		Energy expenditure	
	h	min	Cal./min	Total Cal.
Light sedentary activities	111	19	2·2	14,690
Track walking	15	53	9·4	8,910
In bed	112	58	1·2	8,130
Walking (about 3·5 m.p.h.)	18	11	6·5	7,090
Light standing activities	13	57	4·0	3,350
Dressing, shaving	9	5	4·1	2,235
Lying reading, etc.	18	28	2·0	2,220
Rugby football	2	54	10·0	1,740
Stair climbing	2	22	8·5	1,210
Dancing	2	8	—	800
Stair exercise	1	24	8·4	710
Standing at ease	3	20	2·1	420
Total	13 days			51,510

Table 5. *Energy expenditure of Alistair over 13 days*

Activity	Time		Energy expenditure	
	h	min	Cal./min	Total Cal.
Light sedentary activities	103	46	2·1	13,070
Track walking	17	14	10·1	10,390
In bed	118	16	1·15	8,160
Walking (about 3 m.p.h.)	12	46	5·5	4,080
Light standing activities	14	41	3·8	3,350
Lying at rest	24	49	1·5	2,230
Dressing, shaving	5	49	3·7	1,290
Stair climbing	1	44	9·8	1,020
Sitting at ease	8	54	1·8	960
Stair exercise	1	21	10·3	870
Sundry activities	2	40	—	560
Total	13 days			45,980

Table 6. *Energy expenditure of Evan over 13 days*

Activity	Time h	Time min	Energy expenditure Cal./min	Energy expenditure Total Cal.
Light sedentary activities	90	31	2·0	10,860
Track walking	11	48	10·7	7,580
In bed	121	13	1·2	8,730
Light standing activities	24	7	4·0	5,790
Walking (about 3 m.p.h.)	6	33	5·3	3,990
Lying at rest	36	25	1·6	3,500
Dressing, shaving	7	32	4·3	1,940
Stair climbing	2	44	10·0	1,640
Dancing	2	14	—	910
Stair exercise	—	51	10·4	590
Sundry activities	2	1	—	470
Total	13 days			46,000

Table 7. *Energy expenditure of Iain over 13 days*

Activity	Time h	Time min	Energy expenditure Cal./min	Energy expenditure Total Cal.
Light sedentary activities	126	18	2·3	17,430
Track walking	15	8	10·3	10,100
In bed	117	0	1·2	8,420
Light standing activities	15	28	4·0	3,710
Dressing, shaving	13	43	4·0	3,290
Walking (about 3 m.p.h.)	6	2	5·5	2,000
Stair climbing	2	15	9·3	1,260
Stair exercise	1	29	11·8	990
Dancing	2	14	—	900
Sitting at ease	7	29	2·0	900
Sundry activities	4	54	—	580
Total	13 days			49,580

Table 8. *Energy value of diets* (*Cal.*)

First sedentary period	John	George	Alistair	Evan	Iain
Day 1	2,920	3,140	2,900	2,960	3,050
2	3,300	3,690	3,430	3,580	3,370
3	3,650	4,010	3,540	3,510	3,480
Mean	3,290	3,610	3,290	3,350	3,300
Action period					
Day 4	4,270	4,330	4,530	4,240	4,020
5	3,660	4,040	4,360	3,580	3,670
6	3,540	4,340	4,360	3,560*	3,740
7	1,080†	4,050	3,630	3,320	3,250
8	990†	4,010‡	4,090	3,730	4,430
Mean	3,820§	4,150	4,190	3,690	3,820
Second sedentary period					
Day 9	1,410†	4,370	3,500	4,260	4,040
10	1,800†	4,530‡	3,840	4,150	3,420
11	3,220†	4,480	4,250	3,490	3,520
12	3,550†	4,260	4,450	4,050	3,250
13	2,890†	3,840	4,140	3,700	3,060
Mean	2,576	4,300	4,040	3,930	3,460
Grand total (13 days)	36,280	53,090	51,020	48,130	46,300

* Sedentary. † In bed—mumps. ‡ One game Rugby football. § Average of 3 days only.

Table 9. *Total estimated energy expenditure of the five subjects over 13 consecutive days compared with energy value of the diet for the same period*

	John	George	Alistair	Evan	Iain
Estimated energy expenditure Cal.)	34,310	51,510	45,980	46,000	49,580
Energy value of diet (Cal.)	36,280	53,090	51,020	48,130	46,300
Weight changes (kg)	+0·34	+0·31	+2·66	+0·14	−2·49

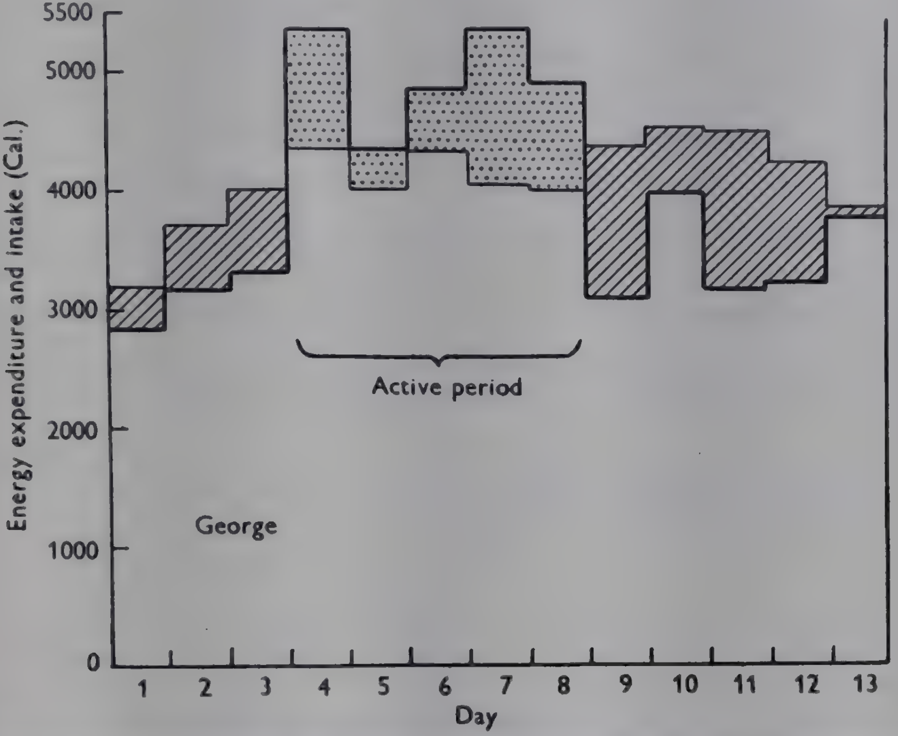

Fig. 1. Energy balance for George. Thin line, daily energy intake; thick line, daily energy expenditure. Cross-hatched areas show excess intake over expenditure; stippled areas show excess expenditure over intake.

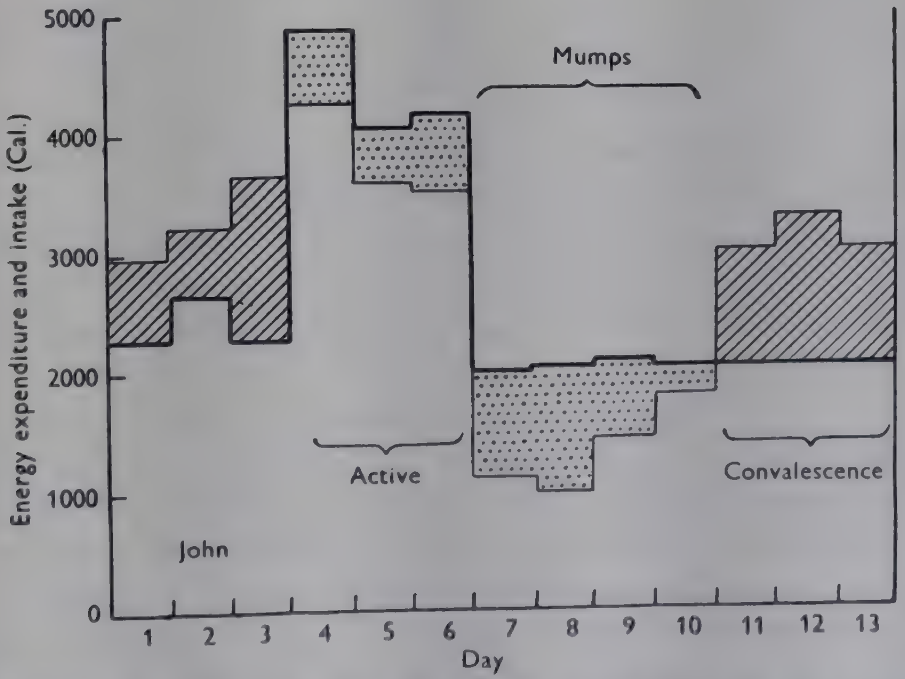

Fig. 2. Energy balance for John. Thin line, daily energy intake, thick line, daily energy expenditure. Cross-hatched areas show excess intake over expenditure, stippled areas show excess expenditure over intake.

explained to some extent by natural relaxation at the end of term, and also perhaps as a compliment to our cook. During the 5-day exercise period, when expenditure rose as high as 6000 Cal., intakes also rose, but never to the level of output. This may be explained by simple physical fatigue. The men were just too tired to eat. The deficit was made good during the second 5-day sedentary period, when all ate each day food with an energy content a little greater than the energy expended. Fig. 1 shows the day-to-day variations in intake and expenditure for George. Alistair, Evan and Iain all showed essentially the same pattern. John also followed the same course until mumps developed on the 7th day. Thereafter, till the end of the experiment, he stayed in bed with a daily expenditure remaining constantly around 2000 Cal. For the first 2 days the fever and the pain restricted food intake to about 1000 Cal., but, on the final 3 days, when convalescence was fully established, he made up the deficits with intakes of the order of 3000 Cal. Fig. 2 shows his two periods of negative balance (exercise and acute illness) and his two periods of positive balance (good appetite and convalescence). By a happy chance these two pairs equalled each other, the weight lost in the illness was regained and over the 13 days a good balance was obtained.

Table 10. *Average energy expenditure (Cal.) of the five subjects during sedentary and active periods compared with recommended allowances*

	John	George	Alistair	Evan	Iain
			Average energy expenditure		
Sedentary period	2400	3240	2870	3040	3080
Active period	4350	4950	4610	4650	5040
			Recommended allowances (Food and Agriculture Organization of the United Nations, 1950)		
Activity: Moderate	3019	3378	3553	3725	3825
			(Canadian Council on Nutrition, 1950)		
Sedentary	2225	2425	2575	2675	2750
Moderate	2750	2950	3100	3200	3275
Heavy	3500	3700	3850	3950	4025
Very heavy	4500	4700	4850	4950	5025

Energy expenditure and reference standards. Table 10 shows the average expenditure of the five subjects during the sedentary and active periods set out in relation to two well-known reference standards. Allowances have been made for the age and weight according to the recommendations laid down by the Food and Agriculture Organization of the United Nations (1950) and the Canadian Council on Nutrition (1950).

It will be seen that, during the sedentary period, the energy expenditure for four of our subjects was between 600 and 900 Cal./day less than the F.A.O. recommendations. Our fifth subject, George, was, however, expending only 150 Cal./day less during the sedentary period than the F.A.O. recommendation based on moderate activity. As has been explained earlier, George normally moved more quickly than the others. During the period of great physical activity the subjects expended between 900 and 1600 Cal./day in excess of the F.A.O. estimate.

Except for George, the energy expenditure during the sedentary period lay between the allowances recommended for sedentary occupation and moderate activity on the

Canadian standard. During the period of great physical activity the energy expenditure of all subjects was within 300 Cal. of that estimated by the Canadian Council on Nutrition for very heavy workers.

DISCUSSION

For practical reasons it was unfortunately not possible to carry on this experiment for longer than 13 days. The inclusion of a water-balance study would have been of considerable value in the interpretation of these results, but was too complicated to include in the programme; over the short period there were differences in the initial and final body-weights, but it is impossible to estimate how far these differences represent changes in body tissue.

Two subjects showed considerable weight changes; Iain lost 2·49 kg and Alistair gained 2·66 kg. The others all gained weight, but less than 0·34 kg. The differences between the energy value of the food eaten and the energy expended were in agreement with these weight changes, being −3300 Cal. for Iain, +5000 Cal. for Alistair and between +1500 and +2100 Cal. for the others. When the experiment had to be terminated at the end of the 13th day, John, Alistair and Evan were still eating in excess of their estimated daily energy expenditure, even after having made up the deficit caused by the very high expenditure between the 4th and 8th days, although it was evident that the excess of intake over output was decreasing fairly rapidly. George, on the other hand, appeared to have reached a balance on the 13th day and it might reasonably have been expected that the others would have done likewise within a comparatively short time. Iain too, was at a fairly constant level during the last 2 days, but it is impossible to translate his weight loss into terms of calories withdrawn from store because of the lack of information on water metabolism.

George, therefore, is the only subject from whose data conclusions based on the intake-output basis can, with any reasonable degree of certainty, be drawn. It should be noted that this subject was the only one other than John (who had to go to bed during the very active period as a result of mumps), who had been taking regular exercise during the winter and was therefore least affected by the strenuous physical activities of the 4th–8th experimental days. For him the difference between intake and output over the whole period was less than 3 %. The same degree of agreement might have been obtained by Evan had the experiment been continued for a longer period, but at the end of the 13th day the difference amounted to 4 %. The net weight difference over the experimental period for all five subjects amounted to less than 1 kg and the estimated energy value of the food eaten exceeded the estimated energy expenditure by 3 %.

The results therefore show a satisfactory degree of agreement between energy intake, as food, and energy expenditure as determined by the Kofranyi-Michaelis analysis of activities. Had the subjects been more accustomed to the types of activity which they carried out between the 4th and 8th days it can be predicted that the day-to-day difference between intake and output would have been lessened. This last condition would be found in a study of subjects on heavy work continuously, as in some industries.

SUMMARY

1. The Kofranyi-Michaelis calorimeter was used to estimate the energy expenditure of five subjects over a period of 13 days, during 5 of which the subjects carried out tasks involving great energy expenditure.

2. The results were compared with the energy value of the food eaten during the same period.

3. A satisfactory agreement between these two estimates over this comparatively short period was found in spite of the fact that three subjects were affected by the severity of the work involved in some of the tasks for which they were not in training.

4. It is suggested that this technique might be used to estimate the energy expenditure of operatives, especially those engaged in heavy industry.

This experiment would have been impossible without the active co-operation of the subjects, Alistair, Evan, George, John and Iain. They cheerfully undertook the labours of walking under adverse conditions and the monotony of note-taking.

Dr H. E. Magee, Ministry of Health, kindly arranged the supply of two calorimeters from Germany.

We are grateful to Dr C. P. Stewart and the staff of the Clinical Laboratory, Edinburgh Royal Infirmary, for analyses of the food. The Edinburgh Royal Infirmary, and the South-Eastern Regional Hospital Board, Scotland, lent us much valuable domestic equipment. Dr B. Woolf and Miss Joyce Thomson advised and helped with the calculations. Finally, we wish to thank the Medical Research Council for a financial grant which covered the cost of the board for the subjects.

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The Effects of Additions of Calcium Carbonate to the Diet of Breeding Mice

1. Effects on Reproduction and on the Heart and Thymus Weights of the Weanlings

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(Received 9 August 1951)

Within recent years several experiments on laboratory animals at this Institute have indicated that additions of calcium carbonate to the diet may have deleterious effects. Thus the effects of pyridoxin deficiency induced in suckling rats by excess of aneurin were accentuated when calcium carbonate was also added to the diet (Richards, 1945); when the amount of added $CaCO_3$ was increased, there was also a retardation in the rate of growth of young rats after weaning (Richards, 1949*a*). Simpson (1947) found that additions of $CaCO_3$ to the diet of adult rats increased iodine excretion and diminished the iodine content and concentration in the thyroid. More recently Howie & Porter (1950) found that a slight modification of the B diet of Sherman gave better reproductive results in mice than another modification in which one of the changes was a 1% addition of $CaCO_3$.

These observations led to the present experiments, of which the initial object was to test the effects on reproduction in mice of including different levels of $CaCO_3$ in the original slightly modified Sherman B diet and at the same time to assess the relative importance of the various changes made in this diet by Howie & Porter (1950). The results of the first experiment indicated that any differences resulting from the other modifications were of minor importance compared with those due to the additions of $CaCO_3$. A second experiment was therefore designed to investigate means by which the deleterious effects of these additions could be counteracted. As the work developed, observations were made indicating that it would be of interest to record the weights of various organs of the young mice and their mothers. These aspects are best considered separately, and the present paper is accordingly presented in two parts: the first deals with reproductive effects and the second with effects on organs.

1. EFFECTS ON REPRODUCTION

Two experiments are reported. Materials and methods were similar for both and are described together, but designs and results are given separately for each experiment.

MATERIALS AND METHODS

Both experiments were done with white 'Swiss' mice of the Institute's stock colony. To supply the breeding females for each experiment sufficient litters containing four females were bred at the same time. They were bred on stock diet (diet 61), which consisted of Rowett Institute stock cubes (diet 1 of Howie & Porter, 1950) with a daily supplement of fresh whole milk given *ad lib.* to the mated pairs of adult mice. Four female mice were allotted to each group in Exp. 1 and eight in Exp. 2 with randomization of litter-mates. Each breeding female was reared on diet 61 to the age of 7 weeks and was then given the appropriate experimental diet a week before being mated with a male of the same age. The males were reared in the same way as the females; mating of litter-mate brothers and sisters was avoided. The method of continuous monogamous mating was used, the effect of the diets on reproduction being judged by the number and weight of the young reared in a given period, as described by Bruce & Emmens (1948). In Exp. 1 the males were removed from the breeding cages after 80 days, and the females were maintained on the diets long enough to rear any litters that had been conceived. In Exp. 2 sufficient time was allowed for the rearing of only two litters. The observations were ended by killing the litters on weaning at 21 days of age. Post-mortem examinations were made as detailed in Part 2 of this paper.

EXPERIMENT 1

The object of Exp. 1 was to test on reproduction in mice the effect of adding three levels of $CaCO_3$ to the slightly modified Sherman B diet used by Howie & Porter (1950)—their diet 2— and to three further modifications of it. Diet 2 consisted of: ground whole wheat 66, dried whole milk 33 and NaCl 1 %. Diet 5—the modified diet that gave poorer reproductive results than diet 2 in their experiments—contained: ground whole wheat 60, casein (lactic, unextracted, Glaxo Laboratories Ltd.) 5, dried whole milk 33, $CaCO_3$ 1 and NaCl 1 %. The most likely causes of the poorer results with diet 5 seemed to be either the addition of $CaCO_3$ or a deficiency of the vitamin B complex. Two factors might have contributed to such a deficiency, namely the reduction in the proportion of wheat and the increased requirement for vitamin B resulting from the addition of protein as casein. These possibilities were therefore considered in planning the experiment.

Design

A factorial design of sixteen squares was drawn up, as shown in Table 1, each square representing an experimental diet. Each of the four rows was built upon a different basal diet. In the first row the basal diet was diet 2. In the second row the basal diet (diet 70) was formed from diet 2 by substituting 6 g maize starch for 6 g wheat, thus reducing the vitamin B content without increasing the protein. In the third row the basal diet (diet 74) was derived from diet 2 by substituting 6 g casein for 6 g wheat, thus reducing the vitamin B content but this time increasing the protein. Rows 1, 2 and 3 were completed by the addition of $CaCO_3$ at the rate of 0·5, 1·0 and 2·0 g/100 g of the respective basal diets. In the fourth row 5 g casein were added to 100 g of diet 2 to form diet 78, and the same additions of $CaCO_3$ were made to 105 g of this basal diet. The number in each square of the plan in Table 1 indicates the number of the diet and is used also to designate the group of experimental animals fed on the diet.

Table 1. *Exp.* 1. *Plan of dietary groups to show factorial design*

(The numbers within the table refer to diets. They are also used in the text to designate the corresponding groups of experimental animals)

Row no.	Composition of basal diet for each row, i.e. the diets for column 1		Amount of added $CaCO_3$ (g/100 g basal diet in rows 1–3; g/105 g in row 4)			
	Constituent	Parts	0 (column 1)	0·5 (column 2)	1·0 (column 3)	2·0 (column 4)
1	Ground whole wheat	66	2	67	68	69
	Dried whole milk	33				
	NaCl	1				
	Total	100				
2	Ground whole wheat	60	70	71	72	73
	Maize starch	6				
	Dried whole milk	33				
	NaCl	1				
	Total	100				
3	Ground whole wheat	60	74	75	76	77
	Casein	6				
	Dried whole milk	33				
	NaCl	1				
	Total	100				
4	Ground whole wheat	66	78	79	80	81
	Casein	5				
	Dried whole milk	33				
	NaCl	1				
	Total	105				

Results

First litters. A summary of the reproductive results for the first litters born to each breeding female is given in Table 2. In the upper part of the table figures are given for the separate groups according to the plan shown in Table 1, and in the lower part the results are summarized in columns according to the $CaCO_3$ additions.

The results for the separate groups show that the effect of the $CaCO_3$ additions was marked. Taking the diets in order of the increasing amounts of added $CaCO_3$ (columns 1–4), the numbers of young weaned were, in row 1: 20, 31, 22 and 16; in row 2: 20, 33, 10 and 10; and in row 3: 14, 27, 29 and 8. (See below for row 4, which is treated separately.) Naturally the total weights weaned depended to a large extent on the numbers weaned, so that for these three rows the total weight weaned was highest in column 2, with a marked falling off in columns 3 and 4. Considering the weight per litter weaned, thus eliminating the effect of litters dying before weaning, the same gradation is apparent in the results, the highest weight being found in column 2, with a marked falling off in columns 3 and 4. In row 4, column 2 shows a lower number weaned and total weight weaned than does column 1, owing to the loss of a complete litter, but the weight per litter weaned is as high as in column 1, and there is the same marked falling off in columns 3 and 4 as in the first three rows. Differences between the rows did not reach conventional levels of statistical significance, and the results may therefore be summarized in columns according to the $CaCO_3$ additions. This is done in the lower part of Table 2. Analysis of these results showed that at the highest level of Ca (column 4) there was a significant decrease in the total weight weaned ($P \ll 0.001$) and in the total number weaned ($P \leqslant 0\cdot001$), and a significant increase in both the number and proportion of deaths ($P < 0\cdot01$). Although the differences in the numbers born at the different $CaCO_3$ levels were not statistically significant, it may be noted that there was some decrease at the two higher levels (columns 3 and 4 of Table 2, lower part). In all the measurements the best results were obtained in column 2, i.e. with a $CaCO_3$ addition of 0·5 g/100 g (or 105 g for row 4) of basal diet.

Table 2. *Exp. 1. Reproductive results for first litters. Each dietary group comprised four females continuously mated*

(See Table 1 for factorial design of experiment)

Statistic	Row no.	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
		0	0·5	1·0	2·0
No. weaned/no. born	1	20/29	31/34	22/31	16/26
	2	20/30	33/33	10/29	10/18
	3	14/33	27/29	29/29	8/31
	4	28/33	24/33	12/26	5/30
No. of litters weaned/no. of litters born	1	4/4	4/4	4/4	4/4
	2	4/4	4/4	2/4	2/3*
	3	3/4	4/4	4/4	2/4
	4	4/4	3/4	2/4	2/4
Total weight weaned (g)	1	122·3	187·3	150·7	91·3
	2	157·7	237·6	84·7	56·8
	3	116·0	215·5	161·9	58·3
	4	211·0	159·2	75·8	26·2
Weight/litter weaned (g)	1	30·6	46·8	37·7	22·8
	2	39·4	59·4	42·4	28·4
	3	38·7	53·9	40·5	29·2
	4	52·8	53·1	37·9	13·1
Totals for rows 1–4:					
Born (no.)	—	125	129	115	105
Weaned (no.)	—	82	115	73	39
Deaths (no.)	—	43	14	42	66
Proportion of deaths (%)	—	34·4	10·9	36·5	62·8
Litters born (no.)	—	16	16	16	15*
Failures to rear litters (no.)	—	1	1	4	5
Total wt. weaned (g)	—	607·0	799·6	473·1	232·6
Wt./litter weaned (g)	—	40·5	53·3	39·4	23·3

* One mated female of experimental group no. 73 failed to conceive because the male was infertile.

Second and subsequent litters. Results are not given separately for the second and subsequent litters, which showed greater variability. In general, the tendency in the later litters on all diets was towards improvement, as has been found in previous work at this Institute with borderline deficiency diets (Richards, 1949*b*), but a few animals that suffered from vaginal prolapse failed to produce any litters after the first. An investigation of these cases indicated that the prolapses should be attributed to genetic rather than dietary causes. Adjustments were therefore made by assuming that the measurements for these animals were equal to the means for their dietary groups.

The complete reproductive results for all litters produced, up to four litters in a few animals, are summarized in Table 3, which gives the figures obtained in the experiments, and in Table 4, which gives the treatment means adjusted for litter-mate effects and for vaginal prolapses. There was a significant decrease in the total weight of animals weaned on diets containing the highest level of $CaCO_3$ ($P=0.001$) and in the total number of animals weaned ($P<0.01$). The average number of deaths on diets with this level of $CaCO_3$ was significantly higher than on diets with lower levels of $CaCO_3$ ($P=0.01$), and the highest proportion of deaths also occurred with the highest $CaCO_3$ level ($P<0.001$). The chief danger periods in the rearing of the young mice were the 1st and 3rd weeks of life, relatively few deaths occurring in the 2nd week. In both the danger periods the number of deaths in column 4 was about four times that in column 2. There was no significant effect on the total number of litters or total number of animals born.

Table 3. *Exp.* 1. *Summary of the reproductive results for the whole period of the experiment (continuous mating for 80 days), arranged in columns according to the* $CaCO_3$ *additions, sixteen females being tested for each level of* $CaCO_3$

	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
Statistic	0	0·5	1·0	2·0
Total young born (no.)	313	307	290	274
Total young weaned (no.)	247	274	217	154
No. of deaths:				
Total	66	33	73	120
In 1st week of life	23	14	48	53
In 2nd week of life	11	2	4	2
In 3rd week of life	32	17	21	65
Total proportion of deaths (%)	21·1	10·7	25·2	43·8
Litters born (no.)	42	37	42	42
Litters reared (no.)	38	36	35	33
Failures to rear litters (no.)	4	1	7	9
Total weight weaned (g)	2065·0	2082·8	1739·1	1144·8
Weight weaned/litter (g)	54·3	57·9	49·7	34·7

Table 4. *Exp.* 1. *Treatment means for reproductive data after adjustment for litter differences and vaginal prolapses*

	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
Mean per mated female	0	0·5	1·0	2·0
Total young born (no.)	19·6	19·2	18·2	17·1
Total young weaned (no.)	16·4	17·8	14·6	9·6
Total deaths (no.)	4·1	2·3	3·8	7·5
Total proportion of deaths (%)	0·21	0·12	0·22	0·48
Litters born (no.)	2·6	2·3	2·6	2·6
Total weight weaned (g)	138·2	135·7	116·8	71·6

EXPERIMENT 2

The significant effects on reproduction resulting from the $CaCO_3$ supplements used in Exp. 1 directed attention to the Ca content of the diets and the possible influence of the calcium:phosphorus ratio. From direct chemical analysis of the individual constituents the Ca and P contents of the diets and their Ca:P ratios were calculated.

The Ca contents of the diets in the four columns of Table 1, taken in order of increasing amounts of added $CaCO_3$, averaged 0·34, 0·54, 0·73 and 1·11 %, and the Ca:P ratios 0·70, 1·1, 1·5 and 2·3. The best results for most measurements were found with the diets quoted in column 2 of Table 1, i.e. with the diets whose Ca content averaged 0·54 % and whose Ca:P ratio was about 1·1, whereas in column 4, which showed the values worst in all respects, the corresponding figures were roughly twice these. Exp. 2 was therefore planned so as to test the effect of reducing the higher Ca:P ratios with a phosphorus supplement. Further, post-mortem examination of the weanlings of Exp. 1 (see Part 2 of this paper) had suggested the presence of anaemia, and this was confirmed by haemoglobin determinations (Greig, 1952). The possibility of an iron deficiency was therefore also tested in Exp. 2.

Design

In Exp. 2 the basal diet chosen was diet 78 of Exp. 1 (Table 1). The breeding mice were divided into eight groups, each containing eight animals. The groups were arranged in four pairs, one group of each pair having per 105 g basal diet a 0·5 g $CaCO_3$ supplement and the other 2·0 g (Table 5). These two levels were chosen because in Exp. 1 they had produced respectively the best and worst results. The first pair, with no addition to the basal diet except $CaCO_3$, thus received the same treatment as groups 79 and 81 of Exp. 1. For the second pair, i.e. groups 89 and 90, the iron content of the diet was increased from 35 p.p.m. (determined spectrographically) to 45 p.p.m. by adding 5·624 mg ferric citrate ($C_3H_4OH(COO)_3Fe . 3H_2O$), equivalent to 1·050 mg Fe, to 105 g basal diet. For the third pair, i.e. groups 91 and 92, 2·87 g sodium dihydrogen phosphate ($NaH_2PO_4 . 2H_2O$) were added per 105 g basal diet, to make the Ca:P ratio of the high-Ca group (group 92) approximately equal to that of group 79. For the fourth pair, i.e. groups 93 and 94, ferric citrate and sodium dihydrogen phosphate were added together in the same amounts as for the two

Table 5. *Exp. 2. Plan showing the calcium and phosphorus contents and the* Ca:P *ratios of the diets*

		$CaCO_3$ (g) added to 105 g basal diet							
		0·5 (column 1)				2·0 (column 2)			
Row no.	Diet	Group no.	Ca (%)	P (%)	Ca : P	Group no.	Ca (%)	P (%)	Ca : P
1	Basal diet no. 78 Ground whole wheat 66 Casein 5 Dried whole milk 33 NaCl 1 Total 105	79	0·55	0·48	1·15	81	1·10	0·48	2·29
2	Diet no. 78 + Fe (10 p.p.m.)	89	0·55	0·48	1·15	90	1·10	0·48	2·29
3	Diet no. 78 + 2·87 g sodium dihydrogen phosphate ($NaH_2PO_4 . 2H_2O$)	91	0·54	1·00	0·54	92	1·07	0·98	1·09
4	Diet no. 78 + Fe (10 p.p.m.) + 2·87 g sodium dihydrogen phosphate	93	0·54	1·00	0·54	94	1·07	0·98	1·09

preceding pairs of groups. The plan of the experiment, showing the Ca and P contents of the diets and their Ca:P ratios, is given in Table 5.

Table 6. *Exp. 2. Total weight weaned and weight/litter weaned for first and second litters*

(See Table 5 for explanation of dietary groups. The nature of the dietary supplements other than Ca is indicated in parentheses below the number of the group)

Lower Ca groups				Higher Ca groups			
No. of group	No. of litter	Total weight weaned (g)	Weight/litter weaned (g)	No. of group	No. of litter	Total weight weaned (g)	Weight/litter weaned (g)
79	1	480·4	60·1	81	1	302·0	50·3
(nil)	2	390·3	65·1	(nil)	2	365·9	52·3
89	1	466·9	66·7	90	1	321·6	64·3
(Fe)	2	510·5	72·9	(Fe)	2	578·8	72·4
91	1	464·7	58·1	92	1	365·9	61·0
(P)	2	458·5	65·5	(P)	2	422·6	60·4
93	1	459·2	57·4	94	1	459·8	57·5
(Fe+P)	2	610·0	76·3	(Fe+P)	2	564·9	80·7

Each group contained eight females continuously mated.

Results

Number born. The addition of Fe, P or the higher level of Ca had no significant effect on the number of young born, either in the first or second litters. Combining the figures for both litters, the average numbers born per litter in the four lower Ca groups, taken in the order of column 1 in Table 5, were 8·8, 8·5, 7·1 and 9·1; and in the corresponding higher Ca groups 7·6, 9·2, 7·7 and 8·1. The total numbers born were 518 for thirty-two mice in the lower Ca groups and 491 in the higher Ca groups with one animal fewer. One mated female in group 81 failed to produce a litter within the time limit of the experiment and was therefore discarded. It was later proved that the male was infertile.

Number and weight weaned. The number weaned and total weight weaned were considerably affected by a number of failures to rear first litters. There were six of these failures among the thirty-one females in the higher Ca groups and only one among the thirty-two females in the lower Ca groups, but the mice that failed with their first litters all succeeded in rearing some of the young in their next litters, so that the total number of deaths did not eventually differ greatly in the two sets of groups, averaging 1·6 per litter born in the lower Ca groups and 1·8 in the higher Ca groups. This failure to rear first litters on the higher Ca diets, followed by an improved performance with their second litters, was also observed in another experiment (unpublished) done at the same time. In the unpublished experiment, seven of eighteen mice on diets containing the higher level of $CaCO_3$ failed to rear their first litters, but five of the seven succeeded in rearing part of their second litters. The incidence of failures to rear first litters on the higher level of Ca is not significant in the conventional statistical sense, but nevertheless appears to be of enough interest to warrant repetition of the experiment with greater numbers. This ability to rear some young in their second litters, although they had failed to do so in first litters, is commonly observed with small laboratory animals under unfavourable dietary conditions. It may be due to the mother's power of adaptation to those dietary conditions, or possibly to her own lessened requirement for some dietary essential.

Because of the number of complete-litter failures it seems best to consider the weights weaned both as total weight weaned and weight per litter weaned. These have been collected in Table 6, in which the results for first and second litters are given separately. Considering the results for the first litters only, the influence of the complete failures is reflected in the total weights weaned. For each of the first three pairs of groups the total weight weaned in the higher Ca group (nos. 81, 90 or 92) was much below that in the corresponding lower Ca group (nos. 79, 89 or 91). For the last pair (nos. 93 *v.* 94), in which there were no complete failures, the weight weaned was the same in both groups. The diet had thus been improved by the addition of both Fe and phosphate. Considering the mean weaning weights of the first litters that were reared (Table 6), the depressing effect of the higher $CaCO_3$ level is still apparent in the first pair of groups (nos. 79 *v.* 81), but this effect has been eliminated by the addition of Fe (groups nos. 89, 90) and also by the addition of phosphate (groups nos. 91, 92) and of Fe and phosphate together (groups nos. 93, 94). For the second litters the weaning weights per litter show an increase in all groups except one, no. 92, where there is almost no change, the improvement being particularly marked in groups nos. 93 and 94, which received supplements of both Fe and phosphate.

2. EFFECTS ON ORGAN WEIGHTS

Marked differences in the weights and general condition of the young mice in the different dietary groups of Exp. 1 led to post-mortem examination of some of the weanlings when they were killed at 21 days of age. This revealed the frequent occurrence of pale speckled livers, enlarged hearts and small thymus glands, and it soon became evident that these conditions were more pronounced on the diets with the higher additions of $CaCO_3$. As an example of these findings, Pl. 1, 1 shows the marked differences in the colour of the livers between a litter of weanlings from group no. 78 (no added $CaCO_3$) and one from group no. 80 (added $CaCO_3 = 1{\cdot}0$ g/105 g of diet 78). Differences in heart and thymus weights of these two litters were also marked, as can be seen from figures given in the description of Pl. 1, 1. For comparison there are included the mean heart and thymus weights for fifteen normal litters on various stock diets outwith the present experiments.

The remaining litters of Exp. 1, which included some of the second and all subsequent litters, were therefore examined more systematically, weights being recorded for thymus, heart, liver, spleen and kidneys of representative mice from each litter.

EXPERIMENT 1

Heart and thymus weights. As the investigation of organ weights was not begun from the start of the experiment, the results are incomplete and are therefore not recorded in detail, but sufficient evidence is available to show that there was a significant effect of $CaCO_3$ in increasing the heart weights of the weanlings. The heart weights of the mothers were not significantly affected. Although the effect of $CaCO_3$ on the thymus weights of the weanlings was not found to be statistically significant when all the results were analysed, the figures for heart and thymus weights of the groups in row 4 (Table 1), in which most litters were available, show the tendency for thymus weights to decrease as heart weights increase with increasing amounts of added $CaCO_3$. The figures are:

Organ weights (mg/100g body-weight) of animals in row 4 of Table 1

	Column 1	Column 2	Column 3	Column 4
Heart	906	959	1418	2133
Thymus	333	329	231	158

Other organs. Analysis of the weights (mg/100g body-weight) of liver, spleen and kidneys of the young mice and their mothers did not reveal any significant effects of calcium or other dietary factors on these organs.

EXPERIMENT 2

Methods

In Exp. 2, post-mortem examinations were made of weanlings from all litters. As a rule four mice (if possible two males and two females) were examined from each litter. Heart weights were recorded for both first and second litters and also for the mothers, and the hearts, together with the median lobes of the livers, were stored in 4 % (w/v) formol-saline (10 parts commercial formalin in 90 parts normal saline) for histological examination (see Greig, 1952). In view of the possibility that the heart enlargement found in these experiments might be due, in part at least, to oedema of the heart muscle, estimations were made of the dry-matter content of the hearts from second litters not required for histology. Since these had been stored in isotonic formol-saline, any effect of the fixative on the weight of dry matter would be negligible, particularly for comparative purposes.

Results

Heart weights of weanlings

Absolute heart weights. In general, the weights of organs have little significance as absolute values and must be related to body-weights, but in this experiment the absolute increases in heart weights for weanlings of the same age in the higher Ca groups were very striking. The mean heart weights and corresponding body-weights for the first litters of all groups are given in Table 7. The table shows the number of litters and also the total number of weanlings included in each group average. When the pairs of groups have almost the same mean body-weight, the effect of the higher level of Ca in increasing heart weight is clearly observed, e.g. groups nos. 79 *v.* 81, 89 *v.* 90, and 93 *v.* 94, the increase being especially marked in the first and third of these pairs of groups. In groups nos. 91 *v.* 92 the effect is even more striking, since the higher Ca group, in spite of having a lower body-weight, yet has a considerably higher heart weight. Diminution of heart weight by Fe is seen by comparing groups nos. 81 with 90, 92 with 94, and 91 with 93. Both Ca and Fe effects were significant ($P < 0{\cdot}001$).

Pl. 1, 2 illustrates this result. The mouse on the left, which may be regarded as a normal weanling, represents a litter from group 89, receiving added Fe; the heart weights and body-weights of the litter averaged 60 mg and 10 g respectively. The animal on the right came from group no. 92, the high Ca plus phosphate group; the heart weights of this litter averaged 131 mg and the body-weights 6·6 g. We have occasionally had a weanling with a heart heavier than that of its mother, although her body-weight was from five to six times as great as that of her offspring.

Table 7. *Exp. 2. Mean heart weights and corresponding mean body-weights of weanlings from first litters.*

(The figures in the table are group averages calculated from the litter means. The nature of the dietary supplements other than Ca is indicated in parentheses after the number of the group: quantities of the supplements as shown in Table 5)

Lower Ca groups				Higher Ca groups			
No. of group	Mean heart weight (mg)	Mean body-weight (g)	No. of litters included in mean*	No. of group	Mean heart weight (mg)	Mean body-weight (g)	No. of litters included in mean*
79 (nil)	57·9	7·78	8 (34)	81 (nil)	87·4	7·82	6 (23)
89 (Fe)	68·4	9·18	7 (28)	90 (Fe)	75·3	9·17	5 (20)
91 (P)	72·5	9·36	8 (34)	92 (P)	85·9	8·79	6 (26)
93 (Fe+P)	51·8	8·08	8 (28)	94 (Fe+P)	69·9	8·15	8 (30)

* Figure in parentheses gives the number of young.

Table 8. *Exp. 2. Mean relative heart weights of weanlings (first and second litters) and mothers; and mean relative thymus weights of weanlings (second litters)*

(The nature of the dietary supplements other than Ca is indicated in parentheses after the number of the group: quantities of the supplements as shown in Table 5)

	Mean heart weight (mg/100 g body-weight)*			Mean thymus weight (mg/100 g body-weight) second litters*
No. of group	First litters	Second litters	Mothers	
		Lower Ca groups		
79 (nil)	754 (8)	790 (6)	492 (8)	358 (6)
89 (Fe)	745 (7)	636 (7)	487 (8)	423 (7)
91 (P)	784 (8)	690 (7)	450 (8)	399 (7)
93 (Fe+P)	645 (8)	621 (8)	462 (8)	439 (8)
		Higher Ca groups		
81 (nil)	1154 (6)	1022 (6)	524 (7)	283 (6)
90 (Fe)	843 (5)	783 (5)	484 (8)	323 (8)
92 (P)	993 (6)	954 (5)	531 (8)	275 (7)
94 (Fe+P)	871 (8)	753 (7)	530 (8)	401 (7)

* Figure in parentheses gives the number of litters or mothers included in the mean.

Relative heart weights. The effects of the dietary supplements on the ratio of heart weight to body-weight are shown in Table 8. By comparing the values for each pair of diets (groups nos. 79 *v.* 81, 89 *v.* 90, 91 *v.* 92, and 93 *v.* 94) it is seen that the higher level of Ca increased the relative heart weight. This effect is significant ($P < 0{\cdot}001$). The effect of Fe in decreasing relative heart weight, both at the lower and the higher

Ca level, is also significant ($P<0{\cdot}001$), and is evident from a comparison of the relevant groups (nos. 79 *v.* 89, 91 *v.* 93, 81 *v.* 90, and 92 *v.* 94). The effect of the phosphate addition on relative heart weight was not statistically significant. Groups nos. 92 and 93, with added phosphate, showed both in first and second litters slight reductions in relative heart weight compared with groups nos. 81 and 89, the corresponding groups without phosphate; but when groups nos. 91 and 94, with added phosphate, are compared with the corresponding groups nos. 79 and 90, the reduction is seen only in the second litters. It may be noted that in all groups except group no. 79 the relative heart weights of second litters were less than those of first litters, that is, they showed an improvement.

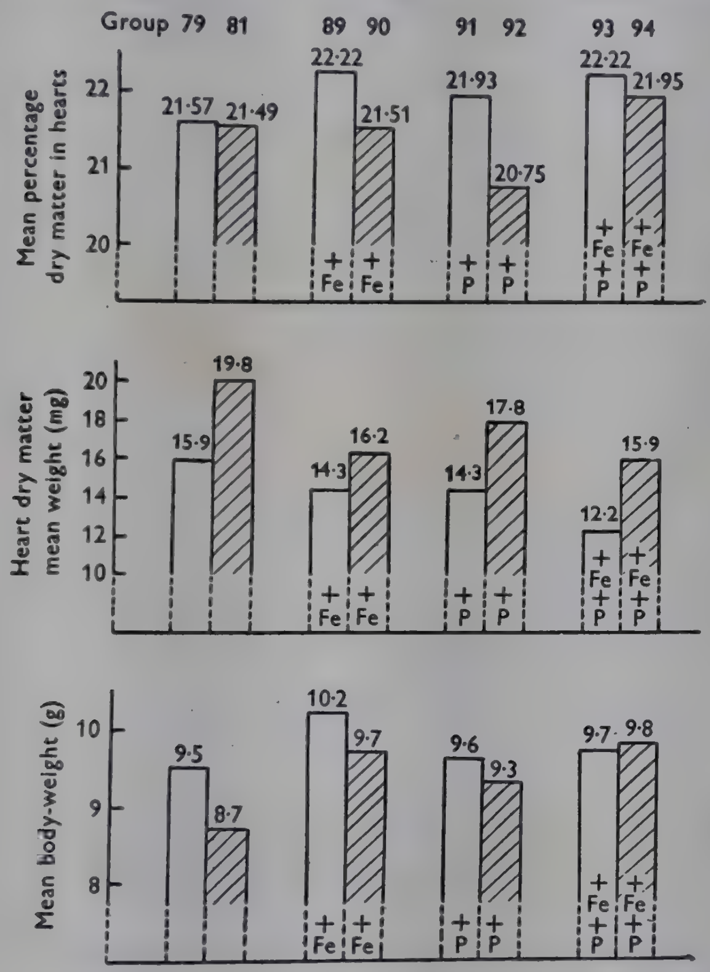


Fig. 1. Exp. 2. Diagram showing mean percentage dry matter in hearts, mean absolute weight of heart dry matter and mean body-weight of second litters. The weight of heart dry matter is greater in the higher Ca group of each pair (shaded columns), in spite of decreased percentage dry matter in heart and diminished body-weight in most animals. For dietary groups see Table 5.

Heart dry matter. The percentage of dry matter in the heart was significantly lowered by the higher level of Ca ($P<0{\cdot}001$) and significantly increased by Fe ($P<0{\cdot}001$), whereas phosphate had no significant effect (Fig. 1). Examination of the results showed, however, that the increased moisture content of the hearts in the higher Ca groups was by no means sufficient to account for the increased weights observed. The weights of dry matter present in the hearts, along with the percentage dry-matter contents and the average body-weights of the different groups, are shown diagrammatically in Fig. 1. The increased absolute weight of dry matter in the hearts of the higher Ca groups is clear.

Relation between heart weights and blood haemoglobin. We have found that a highly significant correlation exists between the heart enlargement in the young mice and the degree of anaemia, as shown by the blood-haemoglobin estimations described by Greig (1952). The closeness of this relationship can be seen from the scatter diagram in Fig. 2, in which Hb is plotted against heart weight expressed in mg/100 g body-weight (H.W. %) for all litters in Exp. 2, irrespective of their group, and for thirty-three litters from the unpublished experiment mentioned in part 1 of this paper. Statistical analysis showed that the relationship between log H.W. % and log Hb was inverse and linear. For the 141 litters observed the correlation coefficient r was $-0{\cdot}936 \pm 0{\cdot}010$, the regression equations for the prediction of one variable from the other being:

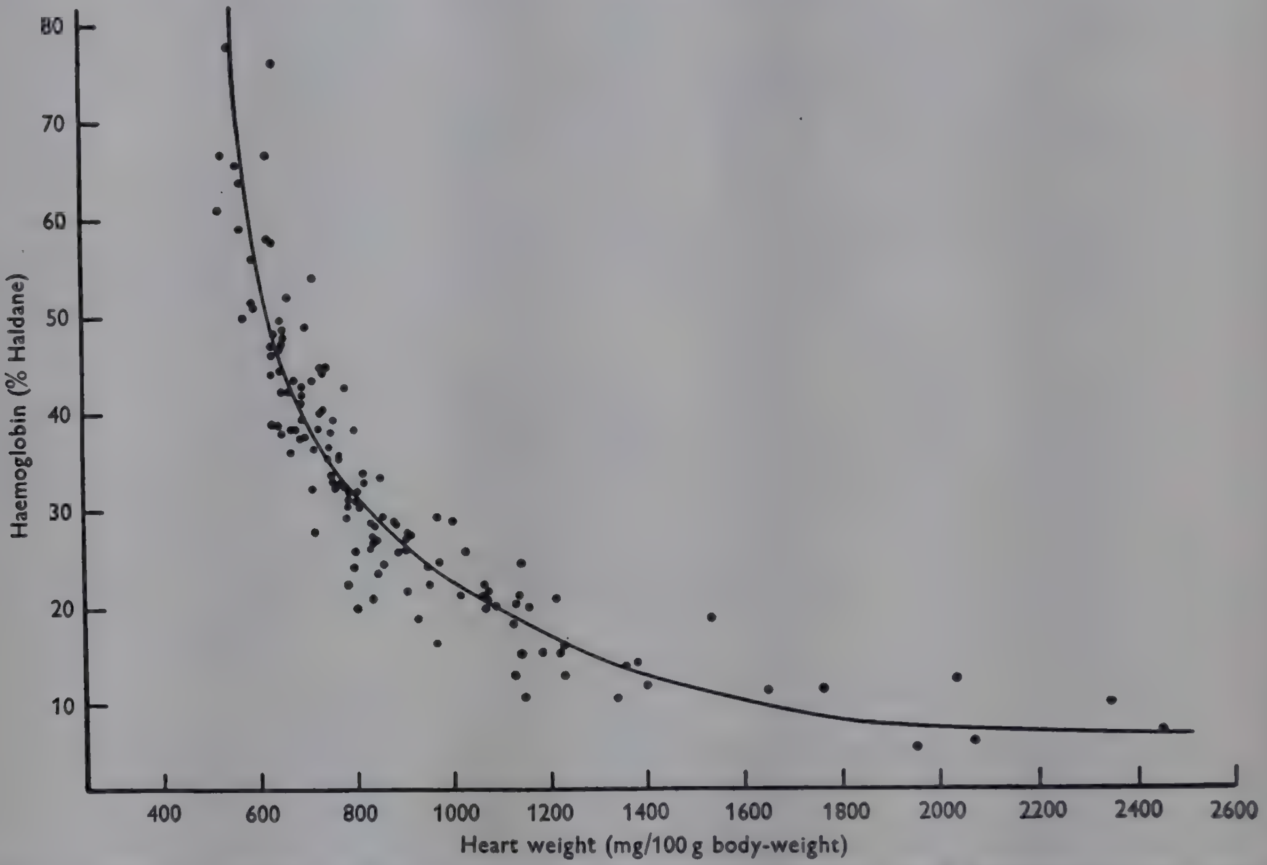


Fig. 2. Exp. 2. Scatter diagram, showing the relationship between blood haemoglobin and heart weight relative to body-weight, for 141 litters irrespective of diet. Each point represents the mean value for one litter.

$$\log \text{H.W.}\ \% = 3{\cdot}780 - 0{\cdot}580 \log \text{Hb} \quad \pm 0{\cdot}018,$$

and

$$\log \text{Hb} = 5{\cdot}955 - 1{\cdot}533 \log \text{H.W.}\ \% \pm 0{\cdot}049,$$

where Hb is expressed as a percentage of the Haldane standard (100 % = 14·8 g haemoglobin/100 ml. blood), and H.W. % = mg heart/100 g body-weight.

Heart weights of mothers

The heart weights of the mothers were also affected by the Ca level of the diet. The higher level of Ca increased absolute heart weight ($P < 0{\cdot}05$), increased heart weight relative to body-weight ($P < 0.001$) and decreased the percentage of heart dry matter ($P < 0.05$). The mean relative heart weights are shown in Table 8. Comparison of the groups shows that the addition of Fe or phosphate did not cause a statistically significant reduction in the heart weights of the mothers. It is possible that the effects would have been more pronounced had the animals been reared on the experimental diets from weaning.

Thymus weights

Thymus weights of second litters were significantly affected both by the Ca and Fe additions, but in the opposite direction from the heart weights. Thus the absolute weight of the thymus and its weight relative to body-weight were both diminished by the higher level of Ca ($P < 0.001$) and increased by the addition of Fe ($P < 0.001$). Again the addition of P had no significant effect. The mean weights of the thymus relative to body-weight are shown in Table 8. The figures show clearly both the diminution in weight in the higher Ca groups and the increase in the groups receiving Fe.

DISCUSSION

In these mouse experiments the adverse effects of $CaCO_3$ on reproduction were seen mainly in a reduction of the numbers weaned, caused by complete or partial failure to rear litters, and in a reduction of the weaning weights of the surviving litters. The number of deaths in the 1st week of life was high. Doubtless some of these deaths, in all groups, must be attributed to causes other than diet, since deaths in the first few days of life are not unusual with small laboratory animals even on normal diets; it is also possible that with the system of continuous monogamous mating the presence of the male may have been an additional disturbing factor. The figures given for Exp. 1 indicate, however, that diet must have been the major cause of these early deaths, since the mortality on the two higher levels of $CaCO_3$ was much greater than on the two lower levels. In the 3rd week the mortality was greatest on the highest level of $CaCO_3$, and it seems justifiable to attribute almost all the deaths during this period to dietary causes. The mother's milk may have been deficient in quality or quantity, or possibly some defect in the young animal may have prevented its proper utilization. This could account both for the large number of deaths during this period and for the decreased weaning weights of the survivors. The most likely explanation of these findings would appear to be an induced Fe deficiency in the mothers, which may have led to a reduced Fe content of the milk, always very low, or to a reduced iron reserve in the young animal at birth.

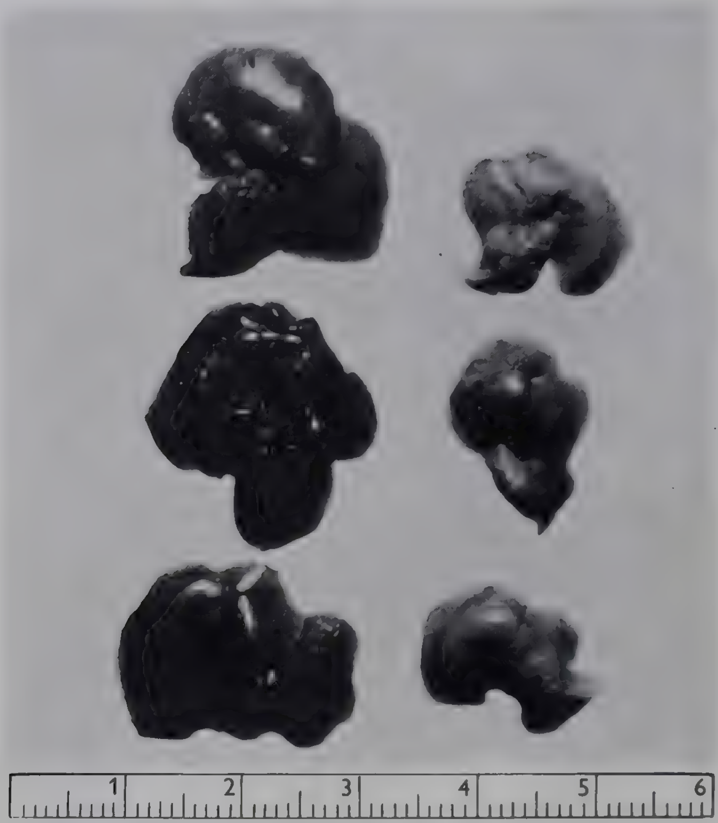
Although the adverse effects of the $CaCO_3$ supplements in Exp. 1 were more apparent on the rearing of the young than on the fertility or fecundity of the mothers, there was, nevertheless, a tendency towards decrease in the numbers born at the two higher levels. The number of animals in each group of that experiment was small, and it seems possible that with larger numbers the decrease in the birth rate might have reached significance. In this connexion it may be noted that in the experiments of Howie & Porter (1950), with twenty-two breeding females in each group, the animals on their diet 2 were not only markedly superior in the number and weight of young weaned to those on their diet 5, but also showed a marked increase in the number of young born. One of the main differences between these two diets was that diet 5 contained 1 % of added $CaCO_3$. The differences in our experiments could perhaps also have been increased by giving the experimental diets to the breeding females at weaning instead of at 7 weeks of age.

As to the mode of action of $CaCO_3$ in impairing reproduction, our results suggest that a dietary Ca:Fe relationship was involved. Excess of Ca seemed to reduce in some way the amount of available Fe, and improvement was effected by increasing the proportion of Fe to Ca, as was shown by the improved weaning weights of the Fe groups, particularly in the second litters. This point is more fully discussed in the second paper of this series (Greig, 1952). To what extent the Ca:P ratio was involved is less clear. Reduction of this ratio by the addition of phosphate did effect some improvement in the average weaning weights of the litters, but to a smaller extent than did the addition of Fe. The effect of P on the organ weights of the weanlings was also much less marked than that of Fe. Thus our results indicate that the Ca:Fe relationship was more important than the Ca:P ratio.

The reproductive findings in these experiments are of interest in view of a recent paper by Hignett (1950), who considers that calcium-phosphorus imbalance in the diet is one cause of herd infertility in cattle. He contends that the $CaO:P_2O_5$ ratio should not be much wider than 1:1, with a sufficient intake of P, and mentions instances of herds in which breeding efficiency was markedly improved by a narrowing of the Ca:P ratio. Shaw (1950) also reported infertility in cows in a district with a hard-water supply, where heavy liming was practised, and stated that a general improvement in the conception rate corresponded to decreased liming.

Our experiments also showed that hypertrophy of the heart and atrophy of the thymus gland were produced in weanling mice by additions of $CaCO_3$ to their mothers' diet. Similar changes were reported by Schmidt (1928) in young mice on a low-iron diet of milk and rice and suffering from a direct iron-deficiency anaemia. He found that the abnormalities could be completely prevented by administering iron in the diet. Since in our experiments the heart and thymus changes could also be prevented by supplementing the mothers' diet with Fe, there seems little doubt that they were associated with an iron deficiency, produced indirectly by the $CaCO_3$ additions to the diet. Thus the heart hypertrophy and thymus atrophy would seem to result from the same essential cause as the impairment in reproduction, namely an imbalance in the dietary Ca:Fe relationship.

The finding that the principal cause of the increase in heart size was an increase in its dry-matter content indicates that a compensatory hypertrophy of the heart muscle resulted from the anaemia. The atrophy of the thymus may have been a consequence of the general under-development frequently observed in the young mice. It is conceivable, however, that the cause of the atrophy may have been purely physical, and that 'accidental involution' resulted from compression of the gland by the enlarged heart. On the other hand, it is known that pyridoxin deficiency causes anæmia in dogs and swine, that it leads to thymus atrophy in young rats and that the effects of



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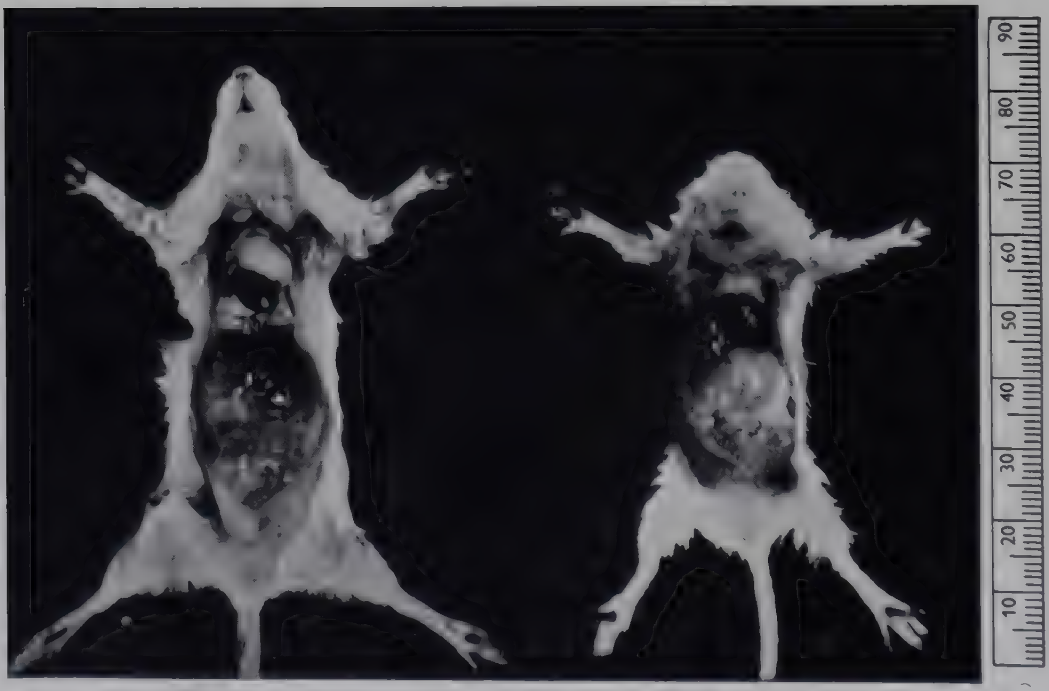
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the deficiency are intensified by additions of $CaCO_3$ to the diet. There may therefore be some more fundamental relationship between the thymus atrophy and the anaemia induced by the $CaCO_3$ additions to the diet.

SUMMARY

1. In a breeding test on mice, using the method of continuous monogamous mating, three levels of calcium carbonate were added to a slightly modified B diet of Sherman and to three further modifications of it. On all the diets the highest addition of calcium carbonate, which gave a Ca intake of 1·1 % and a Ca:P ratio of approximately 2·3, significantly lowered the number and total weight of young weaned and increased the number and proportion of deaths. There was also some decrease in the number of births.

2. When supplements of ferric citrate and sodium dihydrogen phosphate were added to the diet, a number of mothers in the high Ca groups failed to rear their first litters, but in the litters that were reared the weaning weights seemed to be favourably affected by the addition of Fe or by a reduction of the Ca:P ratio.

3. Both absolutely and relatively to body-weight the heart weights of the weanlings were significantly increased by Ca and decreased by Fe, whereas thymus weights were decreased by Ca and increased by Fe. The high Ca level also caused a significant increase in the absolute and relative heart weights of the mothers.

4. Although the percentage of dry matter in the hearts of the weanlings was significantly lowered by the higher level of Ca, there was a marked increase in the absolute weight of heart dry matter at this level, indicating the presence of a cardiac hypertrophy.

5. A highly significant relationship was found between heart weight and blood haemoglobin in the weanling mice, an increase in heart weight accompanying a decrease in Hb. The relationship between log heart weight, expressed in mg/100 g body-weight, and log Hb was linear.

We are indebted to Mr H. M. Quenouille, Statistics Department, University of Aberdeen, for statistical analysis of our data, to Dr R. L. Mitchell, Macaulay Institute for Soil Research, Aberdeen, for spectrographic determinations of iron in the foodstuffs, and to Mr J. Davidson, of this Institute, for their chemical analysis. We wish also to thank Miss Isabel Knowles for assistance in the care and feeding of the animals.

EXPLANATION OF PLATE

1. Exp. 1. Livers of weanling mice showing the effect on liver colour of adding $CaCO_3$ to the mother's diet. (Scale in cm.) Left, livers from group no. 78 (no added $CaCO_3$); right, livers from group no. 80 (1·0 g $CaCO_3$ added to 105 g of diet 78). Mean heart and thymus weights (mg/100 g body-weight) for these two litters and for fifteen normal litters on various stock diets were:

	Heart	Thymus
Litter from group no. 78	952	310
Litter from group no. 80	1987	179
Normal litters	645	471

2. Exp. 2. The weanling mouse on the left from group no. 89, receiving added Fe, shows normal heart, thymus and liver. That on the right from group no. 92 shows the enlarged heart, small thymus and pale liver characteristic of weanlings from mothers receiving excess of $CaCO_3$ in the diet. (Scale in cm.)

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The Effects of Additions of Calcium Carbonate to the Diet of Breeding Mice

2. Haematology and Histopathology

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(*Received* 9 *August* 1951)

Richards & Greig (1952) reported pale livers and enlarged flabby hearts in weanling mice whose mothers' diets contained 1–2 % of added calcium carbonate. It seemed likely that the calcium carbonate had induced an unexpected anaemia in the young.

The work reported in the present paper was undertaken to investigate this possibility and to examine the pathology of the condition. The presence of anaemia was confirmed by haemoglobin estimations, and fuller examination of the blood indicated that it was probably of the iron-deficiency type. A further experiment showed that the anaemia could be prevented by feeding an iron supplement, but not by balancing the additional dietary calcium with sufficient phosphorus to restore the original Ca:P ratio of the diet.

METHODS

The two reproduction experiments reported by Richards & Greig (1952) provided the material for this work. The designs of both experiments have already been described in detail by these authors, and need only brief mention here.

Experiment 1 (Richards & Greig, 1952).

Design. Sixteen litters of four female mice, stratified by weight, were allocated to four Latin squares with litter-mates distributed orthogonally. The other component variables of the squares were the calcium carbonate level of the diet (in vertical columns), and the levels of wheat and of casein in the diet factor (in horizontal rows).

Each diet was a modification of the B diet of Sherman (Sherman & Campbell, 1924). Continuous monogamous mating was allowed for 80 days.

Haematological. All the weanlings in each available litter were examined at 21 days of age. The axillary vessels were resected under ether anaesthesia, and a sample of blood was withdrawn in a Haldane haemoglobin pipette. For a fuller blood examination, approximately 0·5 ml. was withdrawn and heparinized. A drop of fresh blood was used to prepare a blood film, and the animal was forthwith destroyed with ether. All subsequent examinations were performed on the heparinized samples. The mothers were dealt with in the same way, as they became available at the end of the breeding experiment.

Haemoglobin (Hb) was estimated by Haldane's (1901) carboxyhaemoglobin method. Erythrocyte counts (R.B.C.) and leucocyte counts (W.B.C.) were performed in the usual way, using Thoma pipettes and Neubauer haemocytometers, with Hayem's fluid and 1 % (v/v) acetic acid as diluents. Packed erythrocyte volumes (P.C.V.) were determined by means of capillary microhaematocrit tubes, spun for 1 h at 3000 r.p.m. (R.C.F. $=c.\ 1500\,g$). Blood films were stained with Leishman's stain. The same items of apparatus were used throughout the observations, which were made by the same observers. The mean corpuscular haemoglobin (M.C.H.), mean corpuscular haemoglobin concentration (M.C.H.C.) and mean cell volume (M.C.V.) were then calculated from the appropriate observed data.

Histological. The hearts, livers, kidneys, spleens and thymus glands of both litters and mothers were fixed in 4 % (w/v) formol-saline (10 parts commercial formalin in 90 parts normal saline), or in formol-corrosive (10 parts commercial formalin in 90 parts saturated aqueous solution of mercuric chloride) (Lendrum, 1943). Paraffin sections were prepared in the usual manner, and stained by Mayer's (1903–4) haemalum and eosin. Frozen sections, stained with Scharlach R and haemalum, were prepared from the median lobe of the liver of each mother and of a representative of each litter. Smears were made from the femoral bone marrow of a number of weanlings and mothers in various diet-groups and stained with Leishman's stain. In addition, sections of bone marrow were prepared by the method advocated for sternal puncture material by Cappell, Hutchison and Smith (1947); these were stained with haemalum and eosin.

Experiment 2 (Richards & Greig, 1952)

Design. Eight replicates of a 2^3 factorial design were employed, the dietary variables being low or high levels of $CaCO_3$ (0·5 or 2·0 g, respectively, added to 105 g basal diet), and the presence or absence of supplementary iron (10 p.p.m. as ferric citrate) or phosphorus (0·57 % as $NaH_2PO_4.2H_2O$) or both. The sixty-four female mice, in litters of four, were distributed so that two litters were allotted to each block of eight dietary treatments, with the interactions $CaCO_3 \times Fe$, $CaCO_3 \times P$, $Fe \times P$ and $CaCO_3 \times Fe \times P$ partially confounded between litters. Each mother was allowed to become pregnant for the second time before the male was removed from the cage.

Haematological. Each litter was represented, where possible, by two males and two females; these were the two animals nearest to the median weight of the weanlings of each sex in the litter. In general the same procedure was followed as in Exp. 1, but the blood picture of all first litters and mothers was examined. Heller & Paul's (1934) oxalate anti-coagulant mixture was used in place of heparin; leucocyte counts were omitted, but reticulocytes were examined in blood films stained supravitally with brilliant cresyl blue and counterstained with Leishman's stain. Only Hb estimations were done on the whole blood of second litters, but pooled samples of serum from each second litter were used to estimate serum inorganic phosphorus by King's (1946) method.

Histological. Frozen sections of the liver of each mother and of a representative of each first litter were examined.

RESULTS

Haematology

Experiment 1

Litters. Since the haematological observations were begun only towards the end of the experiment, the data were incomplete. No first litters and very few second litters were available, so that most of the observations were on third and fourth litters. Diet-group means were computed by taking the mean for all the young in each litter, and finding from these the mean for all the litters in each diet-group. The means were not weighted for either number in litter or number of litters, and data from second, third and fourth litters were considered together. Statistical analysis of the figures was possible only for Hb; even here the small numbers precluded consideration of each diet-group separately, but the overall effects of modifications in the wheat-casein factor and of changes in the dietary $CaCO_3$ level could be determined by combining, as marginal means, the values in each row (i.e. horizontally) and at each level of $CaCO_3$ (i.e. vertically). The differences between the rows failed, in every instance, to reach significance, but the values for the first (no added $CaCO_3$) and second (0·5 % added $CaCO_3$) levels were significantly greater than the value for the last (2 % $CaCO_3$) level ($P < 0·01$ and $< 0·05$, respectively), whereas values for the third level were intermediate (Table 1).

Table 1. *Exp.* 1 (*litters*). *Mean blood haemoglobin of weanling mice from mothers with four levels of dietary calcium carbonate*

	Percentage of $CaCO_3$ added to mothers' diet*				
	0	0·5	1	2	S.E.
Hb (g/100 ml. blood)	6·96	5·77	4·88	3·85	±0·755

The values at the two lowest levels of $CaCO_3$ are significantly greater than that at the highest level ($P<0·01$ and $<0·05$ respectively).

* In four of the sixteen diets used, additions of $CaCO_3$ were per 105 parts instead of 100 parts basal diet (see Richards & Greig, 1952).

Data on the other attributes are not presented here as they were too limited for a conclusive finding, but in general all measurements, including the calculated M.C.H.

and M.C.H.C. but not, however, the M.C.V., tended to fall in the last two columns, thereby suggesting that the anaemia was hypochromic. This suggestion was borne out by the appearance of the erythrocytes in the stained films. Apart from the polychromatic cells normally found in mouse blood, the red cells of anaemic weanlings were mainly hypochromic, but orthochromatic cells similar to those described by Price-Jones (1932) were also present and stood out in contrast. Anisocytosis was invariable, and poikilocytosis frequent (Pl. 1, 1 *a*)

Mothers. Mean values and standard errors were calculated for each diet-group, row and $CaCO_3$ level, as was done with the litter data. All but six of the sixty-four mothers were available for Hb, R.B.C. and W.B.C. determinations. For these attributes, and for the M.C.H., the arrangement of litter-mates in the design made it possible to isolate and eliminate the component of variability attributable to litter (i.e. genetic) differences. However, P.C.V. estimations could be made on only forty-two animals, and for this attribute and for the erythrocytic indices depending on it, the design had to be treated simply as randomized blocks.

The adjusted results are summarized in Table 2. It will be seen that none of the differences between the rows reached significance; there was not even evidence of consistency between measurements. On the other hand, comparison between the

Table 2. *Exp.* 1 (*mothers*). *Blood picture of mother mice on four basal diets, each with four levels of added calcium carbonate*

(*a*) *Method of calculating marginal means for Hb (g/100 ml.) from diet-group means*

Basal diet†	Percentage of $CaCO_3$ added to diet*				Marginal mean
	0	0·5	1	2	
Row 1	14·19	14·61	13·85	12·71	13·84
Row 2	15·33	12·70	13·68	12·96	13·66
Row 3	15·21	14·33	15·16	12·99	14·43
Row 4	15·36	14·49	13·36	11·84	13·76
Marginal mean	15·02	14·03	14·02	12·62	13·93

S.E. of differences between Marginal means ±0·662
Diet-group means ±1·146

(*b*) *Marginal means for rows, and calcium carbonate levels determined from diet-group means as in* (*a*) *above*

Attribute	Row				Percentage of $CaCO_3$ added to diet*				S.E.
	1	2	3	4	0	0·5	1	2	
Hb (g/100 ml.)	13·84	13·66	14·43	13·76	15·02	14·03	14·02	12·62	±0·662
R.B.C. (10^6/cu.mm)	10·6	10·4	11·5	10·4	11·2	10·3	11·0	10·4	±0·62
P.C.V. (%)	45	41	45	42	48	40	44	42	±3·0
M.C.H. ($\mu\mu$g)	13·2	13·3	12·7	13·3	13·9	13·9	12·9	11·8	±0·92
M.C.H.C. (%)	30·7	34·1	32·3	36·8	35·8	35·8	33·7	29·1	±3·69
M.C.V. (cu.μ)	42·2	40·9	40·2	40·0	43·1	38·8	40·4	40·6	±3·37
W.B.C. (10^3/cu.mm)	3·9	2·4	2·8	2·4	2·4	2·9	2·4	3·7	±3·73

Significant differences:

Hb is lower at 2 % $CaCO_3$ level than at 0, 0·5 and 1 % levels ($P<0·01$, $<0·05$ and $<0·05$).

M.C.H. is lower at 2 % $CaCO_3$ level than at 0 and 0·5 % levels ($P<0·05$ and $<0·05$).

* In Row 4, additions of $CaCO_3$ were per 105 parts instead of 100 parts basal diet.

† For details of the composition of these diets see Richards & Greig (1952).

columns (i.e. levels of $CaCO_3$) showed that the mean Hb of animals on the diets containing the highest level of $CaCO_3$ was significantly lower than that obtained at all other levels ($P < 0{\cdot}01$, $< 0{\cdot}05$, $< 0{\cdot}05$). The mean M.C.H. at the 2 % $CaCO_3$ level was also significantly lower than at the 0 and 0·5 % $CaCO_3$ levels ($P < 0{\cdot}05$, $< 0{\cdot}05$). There was also a suggestion that a similar relationship existed between the M.C.H.C. and the $CaCO_3$ level of the diet. No significant effects were apparent on the other blood attributes.

There was thus considerable evidence that, in the mothers as well as the weanlings, the anaemia was hypochromic. Examination of stained blood films bore this out; the erythrocytes in the more anaemic bloods were pale and presented the same general characters as those of the weanlings.

For further confirmation of these findings, correlation coefficients between Hb and all other attributes were calculated from the data of each individual animal, ignoring treatment effects (Table 3). From these it was clear that Hb was strongly correlated with M.C.H., the relationship being almost linear. This showed that in anaemic animals the haemoglobin carried by each erythrocyte diminished in proportion to the anaemia. The coefficients also confirmed that the blood of more anaemic animals (i.e. those with lower Hb values) had a lower M.C.H.C.; that is to say, the anaemia was hypochromic. No significant correlation, however, could be shown between Hb and M.C.V.; that is to say, the anaemia could not be shown to be other than normocytic. Table 3 also shows that there was a significant correlation between Hb and W.B.C., indicating that the anaemia was accompanied by a leucopenia. Except for the lack of demonstrable change in the M.C.V. (discussed later in this paper) these findings all suggested that the anaemia was of the type associated with iron deficiency.

Table 3. *Exp.* 1 (*mothers*). *Correlation coefficients between haemoglobin and other blood attributes, ignoring treatment effects*

Correlation	r	P
Hb and R.B.C.	+0·460	<0·001
Hb and P.C.V.	+0·667	<0·001
Hb and M.C.H.	+0·531	<0·001
Hb and M.C.H.C.	+0·362	<0·05
Hb and M.C.V.	+0·254	>0·10
Hb and W.B.C.	+0·313	<0·05

Experiment 2

First litters. The summarized results for all haematological attributes measured are shown in Table 4(*a*). The mean value for each litter was first determined, and then the mean of all such litter means in each diet-group. These diet-group means are the figures quoted in the table.

The effects of the various treatments are in most cases obvious from inspection of the data, e.g. the effect of the higher Ca level on the unsupplemented diet is to reduce Hb from 5·00 to 2·66 g/100 ml., and to reduce R.B.C. from 5·4 to 3·4 million/cu.mm, and so forth. The significance of these differences (2·34 and 2·0 respectively) can be determined in each case by using the standard error applicable to comparisons between

Table 4. *Exp.* 2 (*first litters and mothers*). *Diet-group means and standard errors for haematological attributes, and the separate effects of the dietary supplements*

		Hb (g/100 ml.)		R.B.C. (10^6/cu.mm)		P.C.V. (%)		M.C.H. ($\mu\mu g$)		M.C.H.C. (%)		M.C.V. (cu.μ)	
	Supplement†	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
(*a*) First litters (eight litters in each diet-group)	—	5·00	2·66	5·4	3·4	21·5	13·7	9·4	7·6	23·0	18·2	41·2	41·4
	Fe	5·93	4·45	5·9	5·2	24·5	20·4	10·0	8·5	24·0	21·5	41·6	39·9
	P	4·71	3·39	5·5	4·3	20·8	16·4	8·6	7·7	22·5	19·9	38·2	38·6
	Fe+P	6·87	4·00	6·5	4·3	27·4	18·6	10·6	9·1	25·1	20·8	42·6	43·8
	S.E. of difference between means of:												
	Thirty-two litters	±0·31		±0·25		±1·05		±0·40		±0·79		±1·13	
	Sixteen litters	±0·44		±0·36		±1·48		±0·57		±1·11		±1·60	
	Eight litters	±0·63		±0·50		±2·10		±0·80		±1·57		±2·27	
	Effect of:												
	$CaCO_3$‡	−2·00***		−1·53***		−6·26***		−1·44***		−3·57***		−0·03	
	Fe‡	+1·37***		+0·83**		+4·63***		+1·24**		+1·95*		+2·09	
	P‡	+0·22		+0·18		+0·78		+0·07		+0·40		−0·22	
(*b*) Mothers (eight animals in each diet-group)	—	11·93	9·83	9·7	8·5	42·6	37·5	12·4	11·4	28·0	25·7	44·2	44·4
	Fe	14·12	12·70	10·6	10·0	51·6	43·6	13·5	12·7	27·6	28·8	48·9	43·5
	P	14·83	10·74	10·5	9·9	51·1	40·2	14·4	10·8	29·2	26·7	49·0	40·4
	Fe+P	14·43	12·79	10·2	9·4	47·9	44·9	14·1	13·8	30·2	28·6	46·9	48·1
	S.E. of difference between means of:												
	Thirty-two animals	±0·52		±0·39		±1·7		±0·44		±0·55		±1·1	
	Sixteen animals	±0·74		±0·55		±2·4		±0·62		±0·78		±1·6	
	Eight animals	±1·04		±0·78		±3·4		±0·88		±1·10		±2·2	
	Effect of:												
	$CaCO_3$‡	−2·32***		−0·82*		−6·8***		−1·45**		−1·28*		−3·2**	
	Fe‡	+1·67**		+0·42		+4·1*		+1·28**		+1·39*		+2·3*	
	P‡	+1·07*		+0·32		+2·2		+0·77		+1·09		+0·8	

† Supplements per 105 g basal diet: low $CaCO_3$, 0·5 g; high $CaCO_3$, 2·0 g; Fe, 1·05 mg (i.e. 10 p.p.m.); P, 0·57 g as $NaH_2PO_4.2H_2O$. The high $CaCO_3$+P groups and the low $CaCO_3$ without P groups had the same Ca:P ratio, namely 1·0. Basal diet: ground whole wheat 66, dried whole milk 33, casein 5, sodium chloride 1. For full details see Richards & Greig (1952).

‡ In each case means of thirty-two litters or mothers are being compared. Effects were calculated from litter means and are correct to two decimal places, although the diet-group means have been rounded off correct to the first decimal place. Significant effects are indicated thus: *** ($P < 0·001$), ** ($P < 0·01$) and * ($P < 0·05$).

two diet-group means, i.e. the means of two sets of eight litters each. For Hb and R.B.C. these are ±0·63 and ±0·50; thus both differences are highly significant since they are three or four times as large as their respective standard errors. The significance of differences between any two treatments can be determined in this way.

Another estimate of the effect of a dietary treatment can be made by averaging its effects over all other treatments. This estimate is more accurate because it is based on a larger number of observations, and it is valid provided there are no important interactions. For instance, the overall effect of Fe on Hb is

$$\tfrac{1}{4}(5{\cdot}93+4{\cdot}45+6{\cdot}87+4{\cdot}00)-\tfrac{1}{4}(5{\cdot}00+2{\cdot}66+4{\cdot}71+3{\cdot}39)=1{\cdot}37.$$

This difference is more than four times its standard error (i.e. the S.E. of difference between means of sets of thirty-two litters, namely ±0·31) and the difference is therefore highly significant ($P<0{\cdot}001$). Comparisons between means of sixteen litters can be made on the same principle. When the overall effect of the higher $CaCO_3$ level on Hb is determined in this way, it is seen to be more than six times its S.E. ($2{\cdot}00>6\times 0{\cdot}31$). The size, direction and significance of such effects are set out below each attribute in Table 4. The interactions $CaCO_3\times Fe$, $CaCO_3\times P$, $Fe\times P$ and $CaCO_3\times Fe\times P$ were all tested, and in some instances appeared to reach significance at the 5 % level; but as they were small in comparison with the main effects, and inconsistent in their directions, they could safely be ignored in considering main effects.

As can be seen from Table 4(*a*), the overall effect of $CaCO_3$ in reducing Hb, R.B.C., P.C.V., M.C.H. and M.C.H.C. was highly significant. Fe supplementation of the diet also produced significant effects on all these attributes, but in the opposite direction. In strong contrast was the fact that none of the dietary treatments could be shown to produce a significant effect on M.C.V. The effects of P never approached significance.

On this evidence, then, the higher level of $CaCO_3$ in the mother's diet induced a hypochromic, normocytic anaemia, and litters from the non-Fe-supplemented diet-groups also exhibited hypochromic anaemia when compared with the Fe-supplemented groups.

Examination of the supravitally stained smears (Pl. 1, 1 *b*) showed that reticulocytes and late normoblasts were numerous in all specimens. Although systematic differential counts were not made on all smears, these cells appeared to be rather more numerous in the blood of weanlings from the high dietary-$CaCO_3$ groups. In these animals, early normoblasts were also encountered, sometimes in considerable numbers. The mature erythrocytes were hypochromic, and anisocytosis and poikilocytosis were common.

Mothers. The same procedure was followed with the data from the mothers; the results are shown in Table 4(*b*). The differences were not always so large, but the directions of the effects were similar. Here, however, the effect of P on Hb was positive and just reached significance ($P<0{\cdot}05$), and the M.C.V. was significantly reduced by $CaCO_3$ ($P<0{\cdot}01$) and increased by Fe ($P<0{\cdot}05$). The anaemia in the mothers induced by dietary $CaCO_3$ was therefore hypochromic and microcytic, whereas lack of an Fe supplement in the diet also was responsible for a relative anaemia of this type.

The stained blood smears of the mothers presented the same picture as those of the weanlings, except that young normoblasts were unusual.

Second litters. Results for second litters are shown in Table 5. The $CaCO_3$ and Fe effects on Hb were again very strong ($P < 0·001$), and this time the effect of P was also significant ($P < 0·05$). The figures show that, compared with first litters (Table 4*a*), second litters were improved in every diet-group ($P < 0·01$).

Table 5. *Exp.* 2 (*second litters*). *Diet-group means and standard errors for blood haemoglobin and serum inorganic phosphorus, and the separate effects of the dietary supplements*

	Hb (g/100 ml.)		Serum inorganic P (mg/100 ml.)	
Supplement†	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
—	5·77	3·12	11·8	11·5
Fe	7·76	4·53	10·8	11·0
P	6·57	3·70	12·1	10·8
Fe+P	8·52	5·98	12·9	11·2
S.E. of difference between means of:				
Thirty-two litters	±0·40		±0·44	
Sixteen litters	±0·56		±0·62	
Eight litters	±0·80		±0·87	
Effects† of:				
$CaCO_3$	−2·83***		−0·77	
Fe	+1·91***		−0·08	
P	+0·90*		+0·48	

† See footnotes to Table 4.

There were no significant effects on serum inorganic phosphorus. However, a striking hyperlipaemia was observed in serums from the most anaemic litters. This observation is receiving further study.

Histopathology

Experiment 1

Hearts. The hearts both of mothers and of litters from the higher $CaCO_3$-level groups were enlarged (Richards & Greig, 1952). They exhibited marked hypertrophy of the individual muscle fibres and oedematous separation of the muscle bundles. In a number of the most anaemic weanlings from these groups, degenerative changes were apparent in the heart-muscle cells (Pl. 1, 2). Fatty degeneration was never seen, but, in one particularly anaemic weanling, numerous calcified areas were observed scattered throughout the muscle tissue.

Thymus glands. Richards & Greig (1952) found that the thymus glands of litters from the high $CaCO_3$ diet-groups were smaller than those from other diet-groups, but histologically there were no signs of sclerosis or of degeneration in the thymocytes. However, the line of demarcation between cortex and medulla tended to be less distinct, which suggested that the organ had undergone premature involution. No differences were found in the thymus glands of mothers.

Kidneys. The only abnormal features were early degenerative changes in the tubule cells of a few of the most anaemic weanlings. There was no evidence of fatty degeneration.

Spleens. There were no important differences in the structure of the spleens of either litters or mothers on different dietary treatments.

Livers. A certain amount of visible fat was present in sections of the livers of both adult and young mice on even the best diets in this experiment. In a few specimens the quantity of fat was small (Pl. 2, 1), in many it was considerable (Pl. 2, 2), and in some enormous (Pl. 2, 3).

In the weanlings, the impression was gained that the degree of fatty change was greatest when the level of $CaCO_3$ in the mother's diet was highest. In order to corroborate this, the amount of visible fat in comparable sections from a mother and a weanling in each diet-group was assessed by an arbitrary +, + +, + + + system. With only one observation from each diet-group, the results were not suitable for statistical analysis. Nevertheless, they confirmed the impression, since in the weanlings the highest number of ' +'s' was recorded from the highest $CaCO_3$ diet-group, and the lowest number from the two groups with the lowest $CaCO_3$ levels. No association was apparent between the basal diet of each row and the amount of fat present in the liver.

The livers of the mothers were much more variable in their fat content, and no general correlation between liver fat and diet was evident. It was nevertheless true that those livers with the least fat came from mice on the lower $CaCO_3$ diets, and those with the most from animals on the highest dietary $CaCO_3$ level.

The distribution of the fatty changes within the lobule was also studied. When the amount present was small, the fat was generally distributed fairly evenly throughout each lobule (Pl. 2, 1). On the other hand, greater amounts of fat were almost invariably situated in the centrilobular areas (Pl. 2, 2). In the most severe cases, nearly every parenchymatous cell was affected, the appearance being almost that of adipose tissue, relieved only by small patches around the portal vessels (Pl. 2, 3).

The essential lesion in the hepatic cells was fatty degeneration. The earliest changes were manifested in the characteristic 'foam-cells' (Pl. 2, 4), in which the cytoplasm was partly broken down and contained droplets of fat. In more advanced stages, the cells were swollen and contained large vacuoles; and in the most severe cases only the cell wall remained, sometimes with the remains of the nucleus close against it, the cytoplasm having been completely replaced by immense fat globules.

Bone marrow. All the bone-marrow specimens examined were highly cellular, with hyperplasia of the erythroid cell series. The reaction was most marked in marrows from the high $CaCO_3$ groups, which contained an abundance of immature red cells, particularly early and late normoblasts (Pl. 1, 3).

Experiment 2

The procedure described under Exp. 1 (see above) was adopted to assess the amount of visible fat in each of the 128 liver sections. Objectivity in assessment was attained by labelling each slide with a code number which gave the observer no clue to the identity of the specimen, and by recording assessments by two independent observers. The summarized results are presented in Table 6.

Table 6. *Exp.* 2 (*first litters and mothers*). *Diet-group means and standard errors for quantity of visible liver fat, assessed* 1–4 *histologically, and the separate effects of the dietary supplements*

	First litters		Mothers	
Supplement†	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
—	1·6	2·5	2·0	1·2
Fe	0·9	1·9	2·0	2·1
P	1·6	1·6	1·6	2·1
Fe+P	0·8	2·1	1·0	1·9
S.E. of difference between means of:				
Thirty-two animals	±0·26		±0·24	
Sixteen animals	±0·37		±0·35	
Eight animals	±0·53		±0·49	
Effect† of:				
$CaCO_3$	+0·81**		+0·19	
Fe	−0·44		0·00	
P	−0·19		−0·19	

† See footnotes to Table 4.

The effect of the $CaCO_3$ additions to the mother's diet was to increase the amount of visible fat in the livers of weanlings ($P < 0·01$), and there was a suggestion that dietary Fe may have had the opposite effect. The effect of dietary P was not significant, nor were there any dietary effects on the livers of the mothers.

DISCUSSION AND CONCLUSIONS

In these experiments the main haematological findings were clear-cut: calcium carbonate, when added to the diet of breeding mice, induced a marked anaemia in both dams and litters. The immediate cause of the anaemia was undoubtedly an induced iron deficiency, and was evidenced by the hypochromic, anisocytic appearance of the erythrocytes in blood smears, the normoblastic reaction in the bone marrow and the quantitative haematological findings. The anaemia could be prevented by adding ferric citrate to the diet. The effects of the calcium carbonate could not, however, be neutralized merely by restoring the original Ca:P ratio of the diet with a supplement of sodium dihydrogen phosphate.

The anaemia in both weanlings and mothers was characterized by much reduced Hb, P.C.V. and M.C.H. values. The R.B.C. and M.C.H.C. were also decreased, markedly in the weanlings but less so in the dams. Only in the dams, however, did the supplements affect the M.C.V.; this was on the whole raised by Fe and lowered by $CaCO_3$, although the effects were uneven. The relative magnitudes of all effects may be compared in Fig. 1, which has been scaled so that for each attribute the standard errors are represented by equal positive and negative excursions from the base-line.

Discussing the haematology of iron-deficiency anaemia, Parsons (1938) states that, at least in rats, its course is characterized by three stages: there is at first a microcytosis with accompanying mild polycythaemia, then the erythrocytes become hypochromic, and finally erythropenia develops. The syndrome seen in the dams presented all these and many of the other accepted characteristics of iron-deficiency anaemia (see, for example, Whitby & Britton, 1950). The picture was generally similar in the weanlings, except that microcytosis could not be demonstrated, and that changes in the other haematological attributes were more pronounced than in the mothers.

In this connexion, it must be remembered that, as was found by Bacharach, Cuthbertson & Thornton (1949), the effects of iron deficiency are more obvious in weanlings than in their dams. The basal diet in the present experiments contained only 35 p.p.m. Fe (Richards & Greig, 1952), so that animals from all diet-groups were to some extent iron-deficient; indeed, anaemia of the hypochromic microcytic type was already established in litters from even the low-$CaCO_3$ groups, when compared with litters from several stock diets (unpublished observations). The relative differences between weanlings and mothers can therefore be explained on the grounds that the anaemia was much more severe in the weanlings, and consequently the first stage quoted above was not seen.

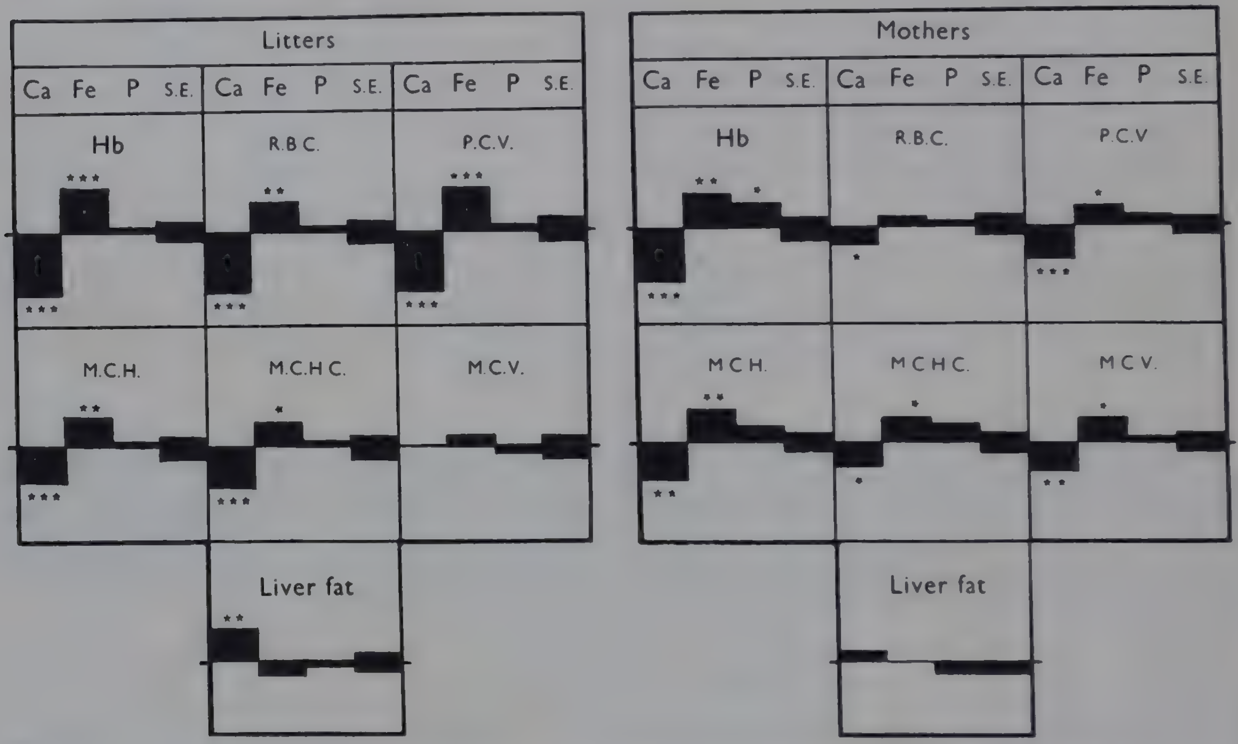


Fig. 1. Exp. 2. Overall independent effects of dietary supplements, to show the relative influence of each on various blood attributes and liver fat. The base-lines represent the overall mean measurements; positive effects (i.e. increases) above the base-line, negative effects (i.e. decreases) below the base-line. The diagram has been scaled so that the standard error is represented in each instance by the same positive and negative excursions from the base-line. Significance of effects is indicated by * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$). Ca = effect of 1·5 % supplement of calcium carbonate; Fe = effect of 10 p.p.m. supplement of iron as ferric citrate; P = effect of 0·57 % supplement of phosphorus as sodium dihydrogen phosphate; S.E. = standard error of the mean.

This explanation might also account for the failure to detect changes in the weanlings' M.C.V.; it would do so if microcytosis were either not progressive in iron-deficiency anaemia or were masked in severe cases by anisocytosis which, as pointed out by Price-Jones (1932), is a feature of this condition. Certainly anisocytosis was well marked in these experiments (Pl. 1, 1). It may have been that a species peculiarity served to aggravate the anisocytosis: polychromatic cells, which are relatively larger than mature erythrocytes, are always numerous in normal mouse blood. Consequently, they may enter the circulation in greater numbers in response to the stress of severe anaemia, although in other species whose blood normally contains only fully haemoglobinized cells, such young cells are seldom mobilized. Although $CaCO_3$ did not reduce the M.C.V., it may not be unimportant that the effect of Fe, which was to raise the Hb and so, presumably, to lessen the anisocytosis, approached statistical significance in raising also the M.C.V. It is thus possible that there were no overall effects on the M.C.V. of the weanlings because macrocytosis in the young cells balanced microcytic changes in the mature erythrocytes.

On the other hand, the estimation of M.C.V. is always subject to considerable technical error. It is calculated from a formula which reflects the errors of two observed measurements; in particular, the notoriously inaccurate R.B.C. is the denominator in the formula, so that random errors in its measurement are greatly magnified in the resultant M.C.V. This is true, of course, for all values of R.B.C.; but when the R.B.C. is small (as it was so often in the anaemic weanlings), not only will errors of the same magnitude affect the M.C.V. to a proportionately greater degree, but substantial errors in its own measurement are more likely. Averages of many observations were taken, and no doubt these errors cancelled themselves out to some extent, but it was clear from the full data for both mothers and litters that variability in the recorded R.B.C. was greatest in the most anaemic animals. This means that much of the variability, and hence error, in the M.C.V. of the most anaemic animals can be ascribed to random variation in the R.B.C. Probably both anisocytosis and random error were jointly responsible for the irregularity observed in the weanlings' cell volume.

The outstanding histological feature was the increased amount of fat in the livers of weanlings from the high $CaCO_3$ groups. Whether this was a direct or an indirect effect of the supplement cannot be stated for certain, but it seems very likely that it was simply a reflexion of the severe anaemia, and was caused by local anoxia. This view is supported by the centrilobular distribution of the fat (areas farthest from the blood supply being most affected) and by degenerative changes in the other tissues, such as heart muscle and kidney tubules, which would probably be affected if the oxygen-carrying capacity of the blood were reduced. These changes did not, however, proceed to fatty degeneration.

Schmidt (1928), working with mice on iron-deficient diets, reported similar histological findings and also observed the associated hyperlipaemia. At the same time, he frequently found fatty deposits, which he regarded as infiltrations rather than degenerations, in the myocardium, pulmonary alveolar cells, and glomeruli of the kidneys, and also centres of calcification in many of the enlarged hearts. Such lesions were not seen in the present experiments, apart from one instance of calcification in the myocardium; but in Schmidt's animals the anaemia was more chronic.

It would seem, then, that the whole syndrome can be explained on the hypothesis that dietary $CaCO_3$ in some way interferes with the availability of Fe, as was postulated by Richards & Greig (1952) for the effects of these diets on reproduction. The addition of phosphate, if it did not much help, certainly did not hinder Fe absorption. Previous work on this subject shows that there is a distinct division of opinion on the effects of dietary Ca salts, and the Ca:P ratio, on Fe absorption.

On the one hand, Kletzien (1935, 1938, 1940) found that, in both rats and mice, many Ca salts reduced Fe storage in the liver, whereas phosphates did not. Shelling & Josephs (1934) and Anderson, McDonough & Elvehjem (1940) also observed that Fe utilization was most efficient when the Ca:P ratio was low. Nordfeldt (1939) reported that calcium-lactate supplements increased the negative Fe balance of mature rats on a cow's milk diet, and Fuhr & Steenbock (1943) found that excessive dietary $CaCO_3$ reduced blood haemoglobin and Fe storage.

On the other hand, the view that low dietary Ca or high dietary phosphate will inhibit Fe absorption seems to have gained wide acceptance (McLester, 1927–49; McCance & Widdowson, 1944; Anonymous, 1950, 1951). It seems to date from the early experiments of von Wendt (1905) and Sherman (1907), and, although their methods were criticized by Davidson & Leitch (1933-4), there is also more recent evidence in its favour. Day & Stein (1938) reported that anaemia developed in rats fed on a high-P diet, unless Ca or some other phosphate-binding ion was also fed; Orten, Smith & Mendel (1936) also found that purified $CaCO_3$ prevented anaemia in rats fed on a diet low in inorganic salts, and Happ (1922) reported that rats on a low-Ca, high-P diet developed anaemia. Further support for this view comes from Kinney, Hegsted & Finch (1949) and Hegsted, Finch & Kinney (1949) who have shown that rats fed on low-P diets absorb excessive quantities of Fe. Again, many workers (e.g. Waltner, 1927; Cox, Dodds, Wigman & Murphy, 1931; Brock & Diamond, 1934; Deobald & Elvehjem, 1935) have demonstrated that excessive dietary Fe causes a negative P balance, presumably by forming an insoluble iron phosphate in the bowel and so preventing proper P utilization. Brock (1937) also showed that dietary Fe, while it increased faecal P, did not affect faecal Ca. It is not unreasonable to argue the converse—that high dietary phosphate will similarly interfere with iron availability—and thence to presume that dietary Ca will increase iron availability by precipitating the P as Ca phosphate.

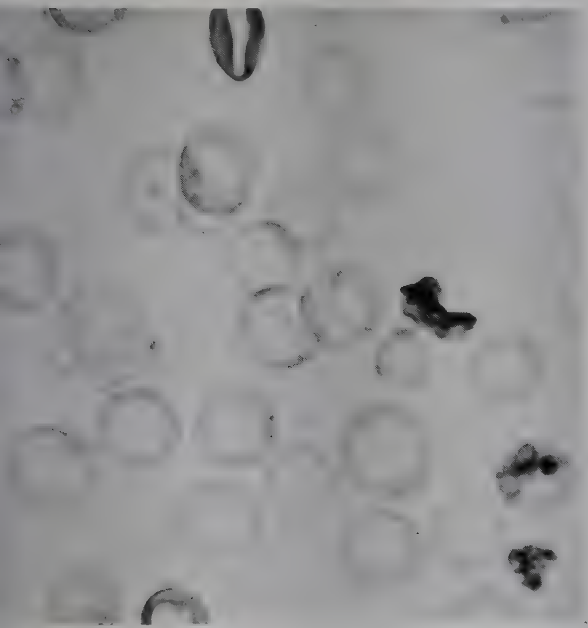
There is thus considerable support for both opinions. The conflicting evidence, together with the findings reported in this paper, indicate that some other important factor, qualitative or quantitative, has not yet been defined.

SUMMARY

1. Weanlings born of white Swiss mice on diets containing 2 % added calcium carbonate were anaemic when compared with weanlings from diet-groups with 0 or 0·5 % added calcium carbonate. The dams on the high-calcium carbonate diets were also anaemic.

2. The addition to the diet of 10 p.p.m. iron as ferric citrate prevented the anaemia, but merely restoring the calcium:phosphorus ratio with sodium dihydrogen phosphate did not do so.

3. Second litters in all diet-groups were less anaemic than first litters.



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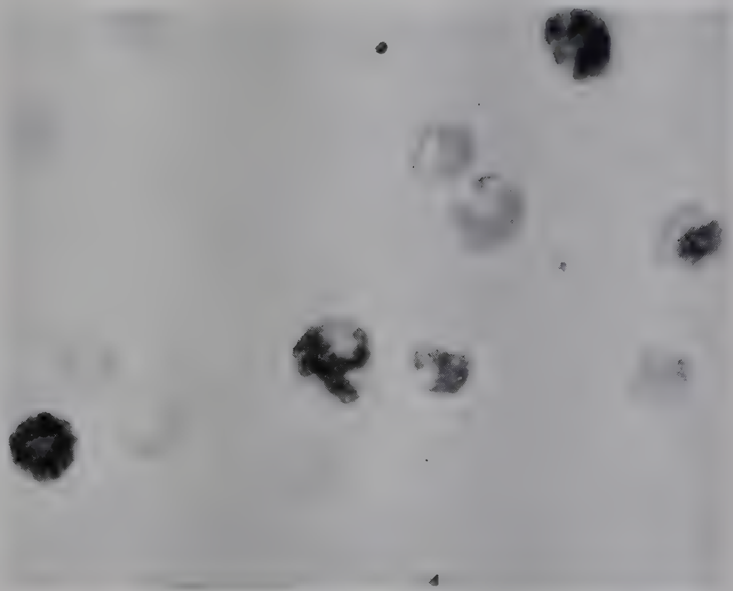

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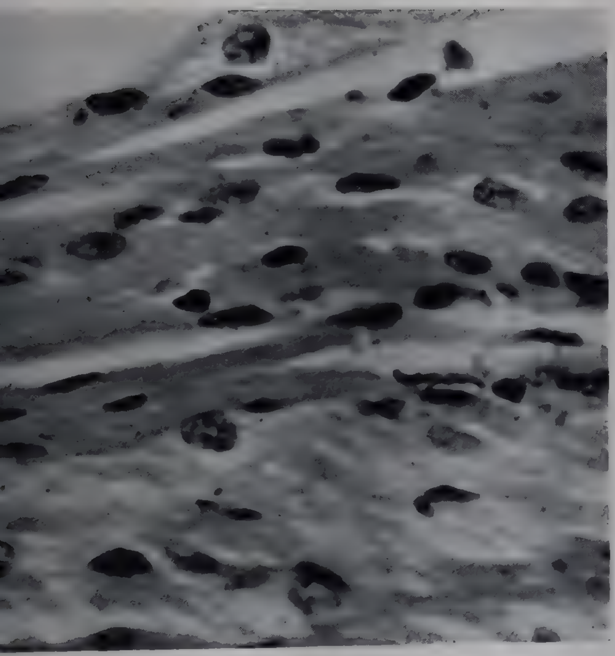

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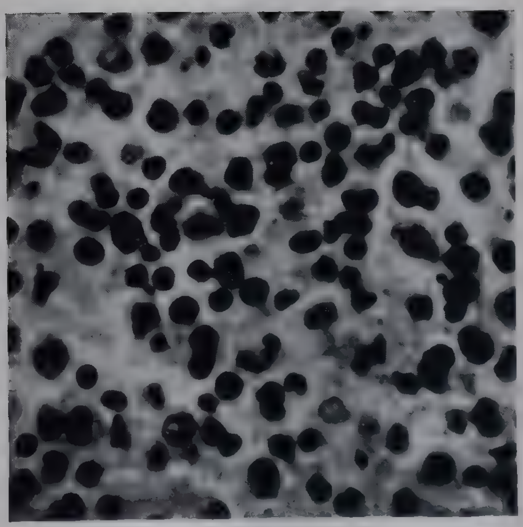

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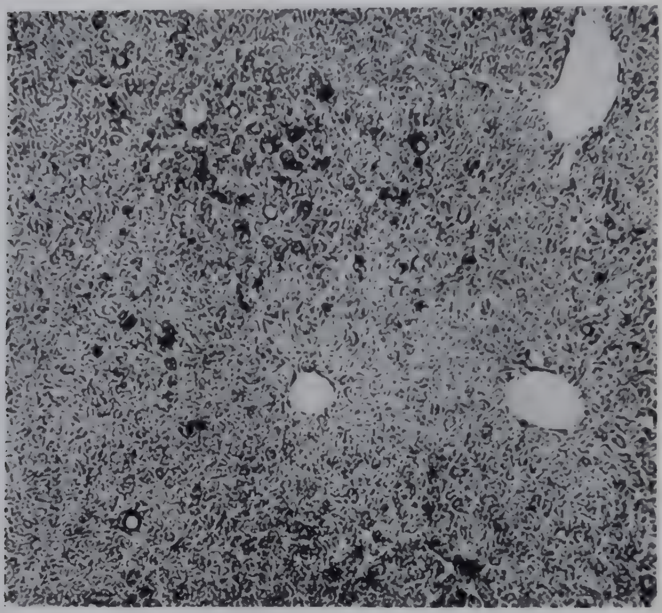

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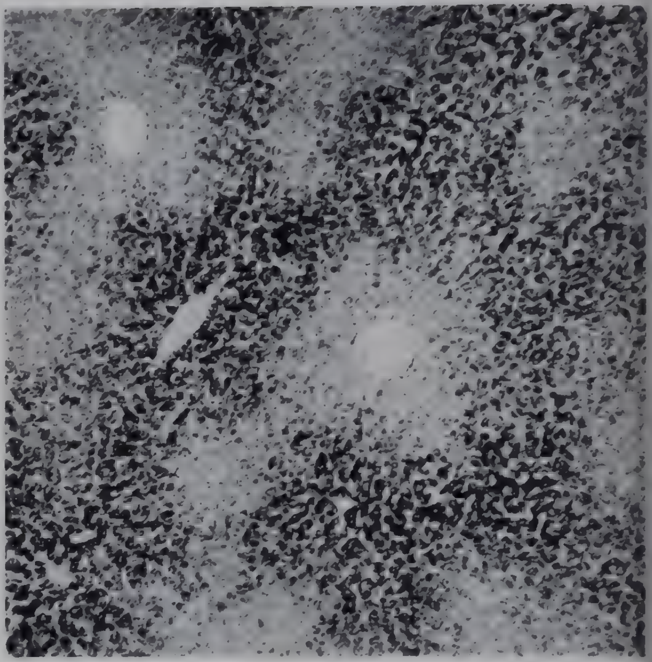

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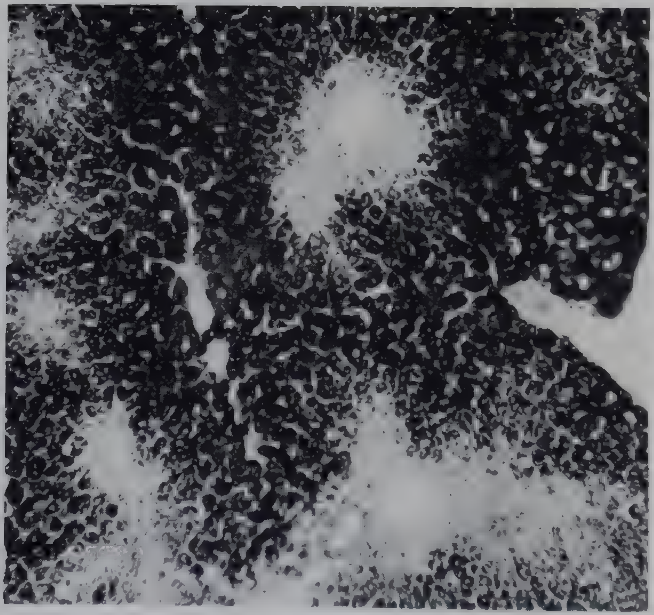

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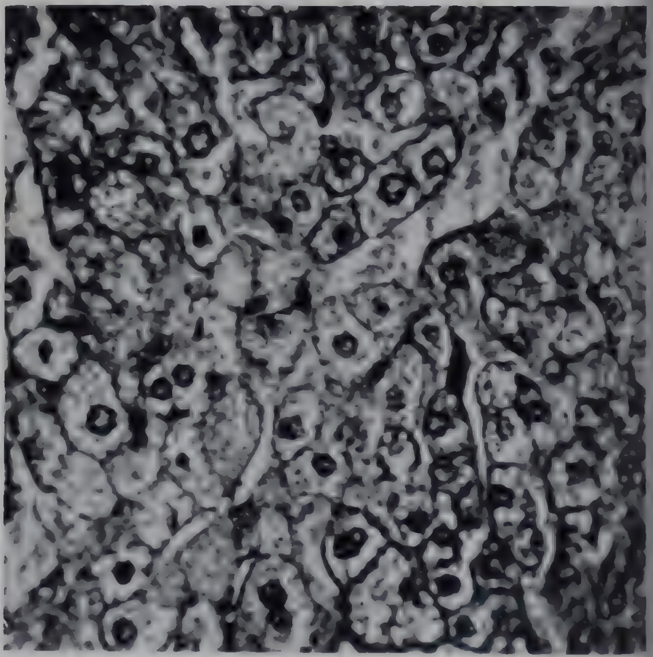

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4. The anaemia in both weanlings and dams presented the haematological features of iron-deficiency anaemia, except that the mean cell volume was not constantly reduced; possible reasons for this are discussed.

5. In association with the anaemia in the weanlings, there were fatty deposits in the liver, marked hyperlipaemia, and sometimes degenerative changes in other organs.

6. Consideration of the literature suggests that some unrecognized factor is important in dietary iron-calcium-phosphate relationships.

I am indebted to Messrs M. H. Quenouille and A. W. Boyne, of the Statistics Departments of the University of Aberdeen and the Rowett Research Institute, for advice on the design of Exp. 2, and for statistical analysis of the experimental data. I am also grateful to Mr Edward Rattray for technical assistance.

EXPLANATION OF PLATES

Plate 1

1. Blood films from weanling mice from high calcium-carbonate diet-groups, *a* stained Leishman, × 1800; *b* stained brilliant cresyl blue supravitally and Leishman, × 1500. Note the hypochromasia, anisocytosis and poikilocytosis, and, in *b*, the normoblasts and reticulocytes.
2. Heart muscle of weanling mouse from high calcium-carbonate diet-group. Note hypertrophy of the fibres and, in the lower half, degenerative changes. Haemalum and eosin, × 700.
3. Section of bone marrow of weanling mouse from high calcium-carbonate diet-group. Note the normoblastic hyperplasia. Haemalum and eosin, × 650.

Plate 2

1, 2 and 3. Livers of weanling mice, containing varying amounts of fat (appears dark). Frozen sections, Scharlach R and haemalum. 1, × 108; 2, × 80; 3, × 95.

4. Liver of weanling mouse, to show the 'foam-cells' typical of the fatty degeneration. Haemalum and eosin, × 400.

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PROCEEDINGS
OF THE NUTRITION SOCIETY

SEVENTY-FIRST SCIENTIFIC MEETING
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE
5 JANUARY 1952

VITAMIN B_{12}

Chairman: PROFESSOR L. J. WITTS, *University of Oxford*

The Discovery and Identification of Vitamin B_{12}

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When the name vitamin B_{12} was proposed by Rickes, Brink, Koniuszy, Wood & Folkers (1948), my colleagues and I debated whether we should adopt the term or retain the earlier name 'anti-pernicious anaemia factor', even when our red crystalline substance was shown to be identical with theirs (Smith & Parker 1948; Fantes, Page, Parker & Smith, 1949). It is now abundantly clear that this substance is indeed properly classified as a member of the B group of vitamins. Moreover, it seems very likely that vitamin B_{12} might have been isolated several years earlier, had this fact been appreciated previously. We were looking not for a vitamin, but for a specific factor concerned in the treatment of pernicious anaemia. Accordingly, the only assay procedures that seemed worth serious consideration were those that attempted to induce in laboratory animals a condition akin to pernicious anaemia. Since this approach failed, in spite of many claims to the contrary, we had perforce to rely on the time-consuming (and inaccurate) clinical test to follow all fractionation procedures. Had we realized that we were looking for a vitamin, then I think we should have tried to develop at a much earlier date appropriate assay methods involving stimulation of growth in deficient animals or micro-organisms. The Americans were evidently less inhibited in their thinking and they did in fact devise a microbiological-assay method that must have been a great help in speeding up their fractionation procedures. It is a little ironical to recall that there are now at least a dozen assay methods for vitamin B_{12}.

Other American workers were indeed well on the way towards the isolation of vitamin B_{12} in the guise of the animal protein factor, using a chick-growth assay method.

It is not necessary to review again the early work on the purification of liver extracts. Our isolation of crystalline vitamin B_{12} from crude liver extracts was only accomplished by the successive application of all, or nearly all, of the following steps: adsorption on activated charcoal followed by elution of impurities with 6 % phenol and of the active principle with 85 % phenol or hot 60 % alcohol; repetition of the adsorption and elution using less charcoal; precipitation of impurities with lead acetate; fractional salting-out with ammonium sulphate; extraction with *n*-butanol from solutions nearly saturated with ammonium sulphate, the active principle being re-extracted from the butanol with water; selective extraction with phenol or cresol mixed with several volumes of butanol or benzene; adsorption chromatography on alumina from aqueous solutions; partition chromatography on damp silica using butanol or butanol-phenol mixtures as mobile phase; repetition of the partition chromatography; adsorption on a small amount of charcoal, followed by elution with 60 % propanol; precipitation with phosphotungstic acid; crystallization from aqueous acetone.

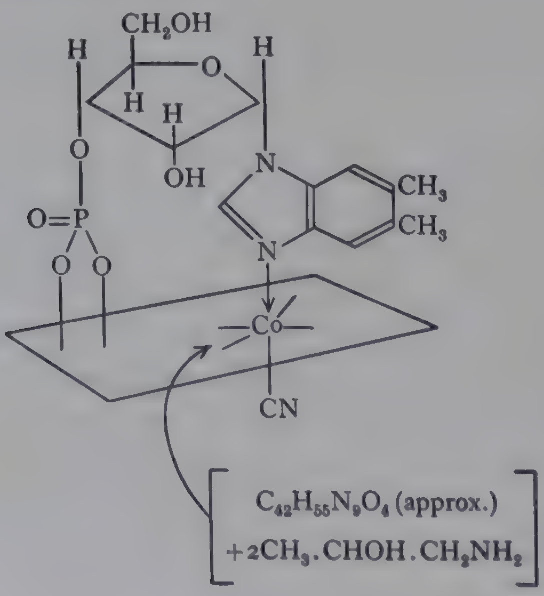
The yields in this multi-stage process were very poor. This can now be partly explained by the fact that we were dealing with a mixture of substances. Some of the initial activity was due to vitamin B_{12b}, and more was doubtless converted into this form by loss of the cyano-group during the processing. The vitamin B_{12b} would be separated from the vitamin B_{12} and lost in some of the fractionation steps.

Vitamin B_{12} is now manufactured by fermentation procedures similar to those used for antibiotics, the usual organism being *Streptomyces griseus*. The fermentation liquors respond to the same fractionation procedures as liver extracts. The following additional methods have mostly been gleaned from the patent literature: adsorption on fuller's earth and elution with solvent mixtures containing organic bases; adsorption chromatography on alumina from solutions in about 75 % methyl or ethyl alcohols; extraction of dry preparations with methanol or ethanol, followed by precipitation with excess of ether.

The procedure can be simplified if traces of cyanide are added from time to time to keep all the activity in the form of vitamin B_{12} itself. For example, I have prepared crystalline radioactive vitamin B_{12} from a few litres of broth containing ^{60}Co by the following steps: adsorption on charcoal; washing with 6 % phenol and elution with 60 % acetone; fractionation with butanol and ammonium sulphate; extraction with a one-to-two mixture of phenol and benzene from alkaline solution; precipitation with ether from this extract and again from an alcoholic solution of the precipitate; crystallization from aqueous acetone.

Chemistry of the B_{12} vitamins

All that is so far known about the constitution of vitamin B_{12} can be represented by the following partial formula:



This shows 5:6-dimethylbenziminazole in glycoside linkage with phosphorylated ribose. All this part of the molecule can be split off by mild acid hydrolysis, leaving a red acidic substance of unknown constitution containing firmly co-ordinated trivalent cobalt. Other hydrolytic fragments are ammonia and two molecules of optically active 1-amino-2-propanol.

Vitamin B_{12} itself has a cyano-group linked to the cobalt atom; the valencies are internally satisfied and the molecule as a whole is neutral, apart from several very weakly basic groups. All the other B_{12} vitamins arise by replacement of CN by other groups and they can all be reconverted into vitamin B_{12} by treatment with cyanide. Thus vitamin B_{12a} (or vitamin B_{12b}) carries a hydroxyl group, or more usually a neutral water molecule, so that the whole molecule becomes basic. It is formed when the cyano group is removed from vitamin B_{12} by exposure to visible light or by treatment with hydrogen and a catalyst or with certain other reducing agents. Vitamin B_{12c} carries a nitrite group, and other acid radicals that co-ordinate readily can be introduced, such as thiocyanate and cyanate. Unstable acidic adducts are known containing two such acid ions (Smith, 1952). The name cyanocobalamin has been proposed for vitamin B_{12} and corresponding names for related compounds.

Physical properties of vitamin B_{12}

Vitamin B_{12} occurs in deep-red crystals containing a variable amount of water of crystallization. It is moderately soluble in water (1·2 %) and in alcohols, but not in most other organic solvents. It is stable in the dry state, even at 100°. Neutral or faintly acidic aqueous solutions can be stored for long periods without appreciable loss, provided bacterial attack is prevented; they also withstand brief autoclaving. Slow destruction occurs at room temperature in solutions more acid than pH 2 or more alkaline than pH 9. Vitamin B_{12} is optically active. It has a characteristic absorption spectrum showing main maximums at 278 and 361 mμ in the ultraviolet and at 525 and 550 mμ in the visible range. The absorption spectrums of the other B_{12} vitamins are similar, but the maximums are slightly displaced.

Bound vitamin B_{12}

In natural sources vitamin B_{12} may occur loosely bound to protein or perhaps to other substances. Some, if not all, of these bound forms are unavailable to microorganisms used for assay purposes. The vitamin B_{12} is easily freed by heat treatment or with proteolytic enzymes.

Combination between vitamin B_{12} and some component of gastric juice can be demonstrated in vitro. Microbiological activity disappears wholly or in part but is largely restored on boiling. The complex is non-dialysable. It has been widely assumed that this 'vitamin B_{12}-binding component' is identical with Castle's intrinsic factor, the substance in gastric juice that promotes the absorption of orally administered vitamin B_{12}. This conclusion appears, however, to be premature, because the two factors exhibit some differences in behaviour and, moreover, binding of vitamin B_{12} can be demonstrated with solutions of some proteins that have no intrinsic-factor activity.

Some new factors closely related to vitamin B_{12} have recently been obtained in pure or nearly pure form, on both sides of the Atlantic (Pfiffner, Calkins, Peterson, Bird, McGlohon & Stipek, 1951; Coates, Ford, Harrison, Kon, Porter, Cuthbertson & Pegler, 1951; Ford, Kon & Porter, 1951; Wijmenga, 1951). Their chemistry has not yet been elucidated, but it has been suggested they may be precursors in the bacterial synthesis of vitamin B_{12}.

Chemical and physical methods of assay

Sufficiently pure concentrates can be assayed directly with a colorimeter or a spectrophotometer. The red cobalt-containing fragment split off on acid hydrolysis can readily be esterified and separated from more water-soluble impurities. It can then be estimated colorimetrically or ashed for colorimetric cobalt determination with one of the nitrosonaphthols. The benziminazole fragment can be removed by drastic hydrolysis and converted into either a coloured or an intensely fluorescent derivative. Finally HCN can be liberated by photolysis and estimated by a sensitive colorimetric reaction with pyrazolone.

Biological assays

Several microbiological-assay techniques have been described, using various lactobacilli or a *Bacterium coli* mutant. Hutner, Provasoli, Schatz & Haskins (1950), who devised an assay method with *Euglena gracilis*, have recently described another with either the Pringsheim chrysomonad strain or *Poteriochromonas stipitata*, which should respond directly to bound forms of vitamin B_{12} (Hutner, 1951).

Assay methods on chicks, rats and mice have also been described. The vitamin B_{12} requirement of these animals can be exaggerated by the use of diets high in vegetable protein or containing thyroxin or thyroid powder. It is usually necessary, or preferable, to minimize the vitamin B_{12} stored by the test animals, by rearing them from females on restricted diets.

Animal and human requirements

It is abundantly clear that vitamin B_{12} is much more than a specific treatment for macrocytic anaemias. It is undoubtedly required in minute amounts by many, if not all, higher animals. The need is difficult to demonstrate in some species, particularly in ruminants, owing to vigorous bacterial synthesis in the rumen or alimentary tract. The human requirement is normally met from dietary sources, although beneficial results have been claimed from administration of vitamin B_{12} in a number of conditions, and in particular for physically backward children. Pernicious anaemia appears to arise not from shortage in the diet, but from impairment of the ability to absorb vitamin B_{12}.

Origin and distribution

Vitamin B_{12} may well be unique in being synthesized exclusively by microorganisms. Its presence in traces in soil, pond water and the roots of some plants could be explained in this way. It has not been demonstrated with certainty in vegetable tissues other than hair roots. The tissues of herbivorous animals probably derive their vitamin B_{12} by absorption of microbially synthesized material from the gut. Carnivorous animals may derive theirs partly in this way and partly from their food.

Vitamin B_{12} has been found in significant amounts in nature only in fermented materials like faeces and in animal products rich in protein. Liver and kidney appear to be the only relatively rich sources and contain around 0·5 p.p.m. Other meats, egg yolk, cheese and casein contain only a few parts per 100 million.

For literature references, see reviews on vitamin B_{12} (Smith, 1950–1; Ungley, 1951–2).

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The Pathogenesis of Megaloblastic Anaemias and the Value of Vitamin B_{12}

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Origins of megaloblastic anaemias

After the discoveries of Minot & Murphy (1926) and Castle (1929) earlier toxic theories were discarded and all megaloblastic anaemias were attributed to either simple or ‘conditioned’ deficiencies of an active principle present in food and stored in the liver. The subsequent isolation of vitamin B_{12}, folic acid and the citrovorum factor further strengthened current beliefs in the purely nutritional origin of this group of anaemias, although dissenting voices had been raised by Dock (1938) and Bomford (1946). In this paper nutritional and toxic theories are welded into a single working hypothesis.

The main dietary sources of vitamin B_{12} are of animal origin: organ meats, muscle meats, fish, milk and eggs. The folic-acid group of substances (including citrovorum factor) comes not only from animal sources but from fruits and green leaves. Yeast is a source of the folic-acid group and of the hypothetical Wills's factor, if this is a separate entity. Moreover, although lacking vitamin B_{12}, some yeast extracts have the properties of an extrinsic factor of Castle in that their haemopoietic effects in pernicious anaemia are potentiated by the simultaneous oral adminstration of a source of intrinsic factor (Strauss & Castle, 1932; Ungley & Moffett, 1936). A deficient intake of these haemopoietic factors or of their precursors is common in the megaloblastic anaemias of tropical and subtropical regions, and not unknown in temperate zones. The low intake may be due to poverty or ignorance, to peculiar food habits based on fads or fancies, religious beliefs, prescribed diets or real or imagined association of symptoms with the eating of certain foods.

Absorption may be impaired by abnormalities in the alimentary tract. Gastric atrophy causes permanent lack of Castle's intrinsic factor in Addisonian pernicious anaemia. The secretion of this gastric factor may be temporarily reduced, e.g. during pregnancy (Strauss & Castle, 1933). The part played by structural and biochemical abnormalities in the intestine and by micro-organisms and helminths is discussed later.

Utilization may be interfered with by infection or by damage to vital organs such as the liver. Extreme deficiency of one haemopoietic factor may interfere with the utilization of another (see p. 303). Demands for haemopoietic factors may be increased by pregnancy, hyperthyroidism or infection and perhaps by prolonged excessive haemolysis, as in acholuric jaundice or malaria. Sometimes there is a vicious cycle, deficiency leading to anorexia, vomiting, diarrhoea or other alimentary disturbances, which in turn accelerate depletion of the nutrient.

Several facts, some clinical or biochemical, others deriving from experiments with animals or marrow culture, are difficult to reconcile with these simple nutritional concepts. For example, certain manifestations of megaloblastic anaemia are more readily explicable on a toxic than on a purely nutritional basis (Ungley, 1950*b*, 1951–2). One recalls the pyrexia that subsides dramatically after therapy, even before the red-cell count has risen appreciably, the patchy and sometimes perivascular distribution of degeneration in the nervous system, and the vivid red patches of glossitis. Sometimes haemolysis is excessive and even red blood cells transfused from normal donors are rapidly eliminated. In five out of six such cases the cessation of excessive haemolysis and the change to a normal rate of elimination occurred within 2 weeks of the administration of vitamin B_{12} or folic acid (Ungley & Walker, unpublished).

Serum from patients with pernicious anaemia in relapse inhibits the maturation of megaloblasts in marrow culture (Rusznyák, Löwinger & Lajtha, 1948; Lajtha, 1950). In vitro the inhibitor can be overcome by adding enough folic acid, citrovorum factor, normal serum, or vitamin B_{12} with gastric juice. During remission the serum ceases to inhibit the maturation of megaloblasts in marrow culture. All these findings have been confirmed in my laboratory either by Thompson (1950) or by Cox (to be published). The nature and origin of the inhibitor are unknown. Its presence in cerebrospinal fluid suggests that it may be ultrafiltrable. It was not destroyed by heating for from 1 to 2 h at 56° (Lajtha, 1950).

The urinary excretion of certain phenols is excessive in relapse and falls to normal after therapy with liver extract (Swendseid, Wandruff & Bethell, 1947) or vitamin B_{12} (Abbott & James, 1950). Another possible toxic agent is indol, which produced haemolysis and anaemia in dogs on a diet deficient in the vitamin B complex, but not in dogs on a normal diet (Rhoads, Barker & Miller, 1938).

In animals neither deprivation of vitamin B_{12} nor complete gastrectomy leads to megaloblastic anaemia. Yet megaloblastic anaemia can be produced both in man and in animals by operations or diseases that lead to stenosis, blind loops or anastomoses in the gastrointestinal tract. All these conditions encourage the growth in the small intestine of micro-organisms which normally flourish in the colon.

Megaloblastic anaemia associated with intestinal strictures and blind loops

The essential feature of such anaemia is an area of stagnant and infected small intestine. When Watson, Cameron & Witts (1948) made culs-de-sac in the intestines of rats, the onset of anaemia was preceded by a long latent period. Schofield, Cox and I are repeating this work using larger animals so as to permit sampling of the blind sac. Our hypothesis is this. A toxic factor or inhibitor is formed by bacterial action in the stagnant area. In the initial stages this inhibitor is detoxicated by postulated enzymes of which folic acid or vitamin B_{12} or both form a part (see below). The increased call for detoxication leads to an increased demand for one or both vitamins, stores of which are gradually depleted. When depletion reaches a certain level, detoxication fails and the animal suddenly becomes anaemic and ill. The experimental anaemia can be prevented or alleviated by the administration of antibiotics (Toon & Wangensteen, 1950; Witts, 1951; Watson & Witts, 1952). In the rat, folic acid was effective, but not liver extract or vitamin B_{12}. In man, subacute combined degeneration may develop and responses to vitamin B_{12} have been observed. Two examples may be cited from a larger series (Thompson & Ungley, to be published).

In Mr C., with intestinal stenosis, the anaemia was initially hypochromic and responded to iron. Later the anaemia became macrocytic and responded to liver extract (see Fig. 7 in Ungley, 1938). In the latest relapse 80 μg vitamin B_{12} produced an effect equivalent to the response expected from about 20 μg vitamin B_{12} in Addisonian pernicious anaemia. At autopsy there was a stagnant and infected area of small intestine.

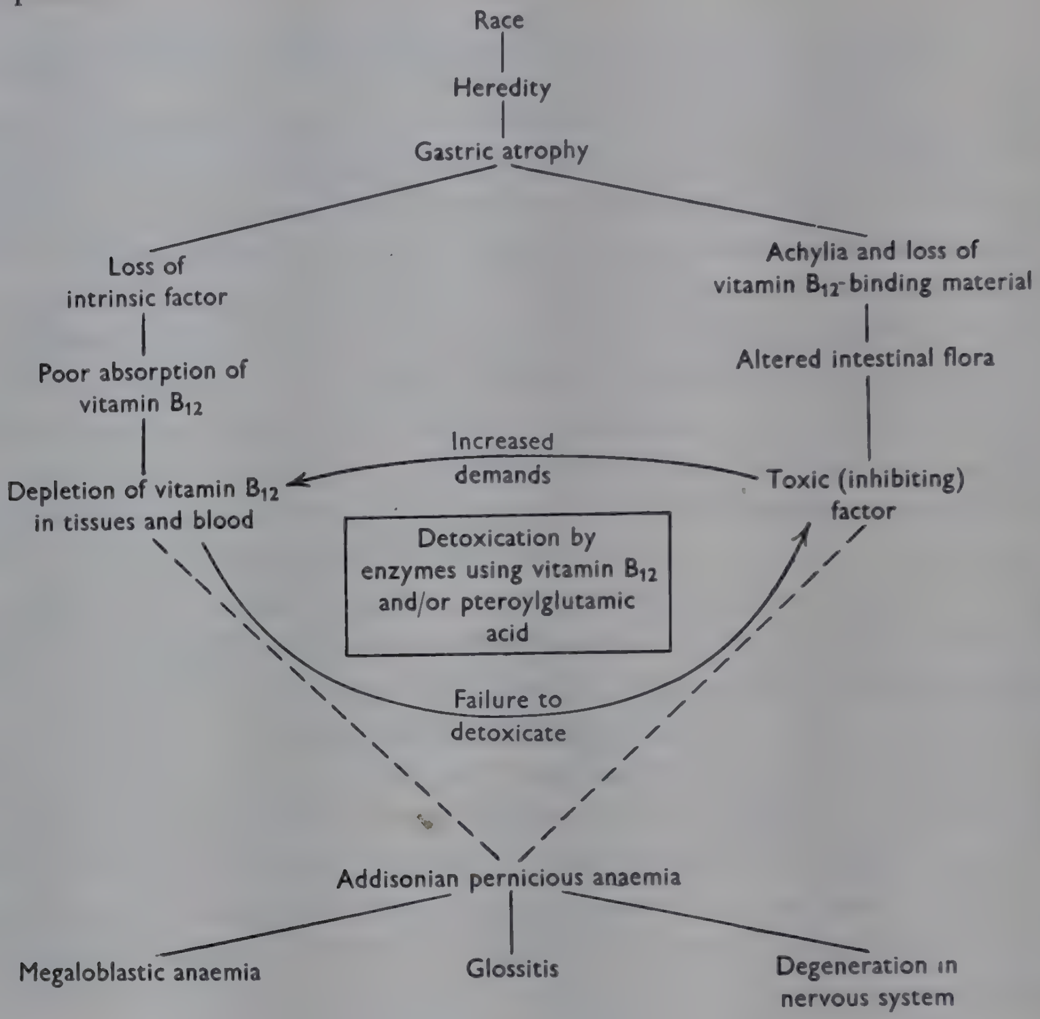
In Mr H., who had had multiple operations on the intestine for Crohn's disease, oral administration was ineffective at first, but after parenteral therapy had restored normal blood values, daily doses of 100 μg vitamin B_{12} orally were enough to maintain remission. In relapse the serum showed an inhibiting effect on the maturation of megaloblasts in marrow culture (Thompson & Ungley, to be published).

Addisonian pernicious anaemia

Lesion. The essential underlying lesion is gastric atrophy. The atrophy is confined to the body of the stomach (corpus, fundus). It involves not only the mucosa but the underlying muscle coats (Magnus & Ungley, 1938). I suggest that this peculiar lesion may be genetically determined; the individual is born not with gastric atrophy, but with a tendency for the stomach to atrophy later in life. Of those whose stomachs atrophy probably all have achlorhydria, but only some develop pernicious anaemia, and then probably after a long interval. Others develop a simple iron-deficiency anaemia, or never become anaemic at all. Similar remarks hold true for the subjects of total gastrectomy.

My working hypothesis is that when gastric atrophy leads to pernicious anaemia it does so by two pathways:

(*a*) Loss of Castle's intrinsic factor leads to defective absorption of vitamin B_{12} and so to depletion.


Race
Heredity
Gastric atrophy
Loss of intrinsic factor
Achylia and loss of vitamin B12-binding material
Poor absorption of vitamin B12
Altered intestinal flora
Increased demands
Depletion of vitamin B12 in tissues and blood
Toxic (inhibiting) factor
Detoxication by enzymes using vitamin B12 and/or pteroylglutamic acid
Failure to detoxicate
Addisonian pernicious anaemia
Megaloblastic anaemia
Glossitis
Degeneration in nervous system

Effect of:			
Vitamin B_{12}	Relieves	Relieves	Arrests
Pteroylglutamic acid	Relieves initially	Relief absent or transitory	No arrest
		May be exacerbation	
Citrovorum factor	Relieves initially at least	?	?

Fig. 1. Suggested pathogenesis of Addisonian pernicious anaemia (working hypothesis only).

(*b*) Loss of free hydrochloric acid reduces the antiseptic action of gastric juice. The contents of the upper intestine lack peptic predigestion. Moreover, the small amounts of gastric juice with which the food is admixed are deficient in substances that bind vitamin B_{12} and render it unavailable to certain bacteria. In this abnormal medium micro-organisms flourish and produce the postulated toxic or inhibiting factor (Fig. 1). In the diagram these two pathways are linked by enzyme systems in which vitamin B_{12} and the folic-acid group of vitamins play an important part.

These vitamins are known to be concerned in methylation and in nucleic-acid metabolism. Here we postulate another, but possibly related function, namely to detoxicate or otherwise eliminate the inhibitor. One recalls the protective action of vitamin B_{12} against poisoning by pyridine (Dinning, Keith, Parsons & Day, 1950) and by carbon tetrachloride (Koch-Weser, Szanto, Farber & Popper, 1950). Day, Hall & Pease (1949) have demonstrated in vivo an effect on the methylation of guanidoacetic acid in rats. Vitamin B_{12} has also been shown to prevent uraemia (and high arginase activity in the liver) in newborn rats from mothers maintained on all-vegetable rations (Liener & Schultze, 1950).

Where then do folic acid and the citrovorum factor fit into this scheme? Why should the giving of folic acid relieve the anaemia, at least temporarily, and yet leave the tongue and the nervous system unprotected or more vulnerable? Conceivably, the giving of an excess of folic acid facilitates the action of the (postulated) vitamin B_{12}-containing enzyme, but in doing so it hastens the depletion of traces of vitamin B_{12} remaining in the body. When these are exhausted folic acid is without effect. Relapse follows, which may be neurological, lingual or haematological. The citrovorum factor is probably a stage in the normal metabolism of folic acid. Conversion of pteroylglutamic acid to citrovorum factor is aided by ascorbic acid, and also apparently by vitamin B_{12} (Dietrich, Monson & Elvehjem, 1951). In marrow culture—but not clinically, except in scorbutic monkeys—citrovorum factor is about 200 times more active than pteroylglutamic acid (Callender & Lajtha, 1951*a*, also personal communication).

In Addisonian pernicious anaemia the sole limiting factor seems to be vitamin B_{12}, although in non-Addisonian anaemia multiple deficiencies are probably not uncommon. In such cases a response to one haemopoietic factor may be inhibited by lack of another. Here are two possible examples (see Figs. 2 and 3). Both resembled Addisonian pernicious anaemia in having histamine-fast achlorhydria and a continued need for maintenance therapy, but one had syphilis and the other had steatorrhoea.

Mr C. (Fig. 2), aged 59 years, had anorexia, vomiting, weakness, dyspnoea, pallor, a smooth tongue and achlorhydria. Treatment for syphilis for 5 years resulted in serological negativity 8 months before admission. Stomach and duodenum were radiologically normal and diet was normal. Values for calcium, phosphorus, proteins, carotenoids and ascorbic acid in the blood spoke against a malabsorption syndrome. With serum bilirubin, 0·8 mg/100 ml., the direct reaction was negative. The marrow was megaloblastic. Red blood cells numbered 1,260,000/cu.mm, haemoglobin was 4·7 g/100 ml., packed cell volume 14 %, mean corpuscular volume 111·1 μ^3, mean corpuscular haemoglobin 37·7 $\mu\mu$g, mean corpuscular haemoglobin concentration 33·9 %, reticulocytes 0·6 %, white blood cells 2000/cu.mm. Transfusion was followed by a control period of 19 days, during which the highest reticulocyte level was 2·6 % and red-cell count fell to 1,300,000. The injection of Smith's slow-moving red component, a concentrate of vitamin B_{12b} (Smith, 1948; Fantes, Page, Parker & Smith, 1949), in a dose colorimetrically equivalent to 10 μg vitamin B_{12} was followed by an insignificant reticulocytosis of 2·8 % without improvement in the anaemia. After an injection of 10 μg ordinary vitamin B_{12} (cyanocobalamin) reticulocytes rose to 6 % on the 5th day, again without improvement in the anaemia. The marrow was still megaloblastic. In the next 6 days a total of 15 mg folic acid was administered orally.

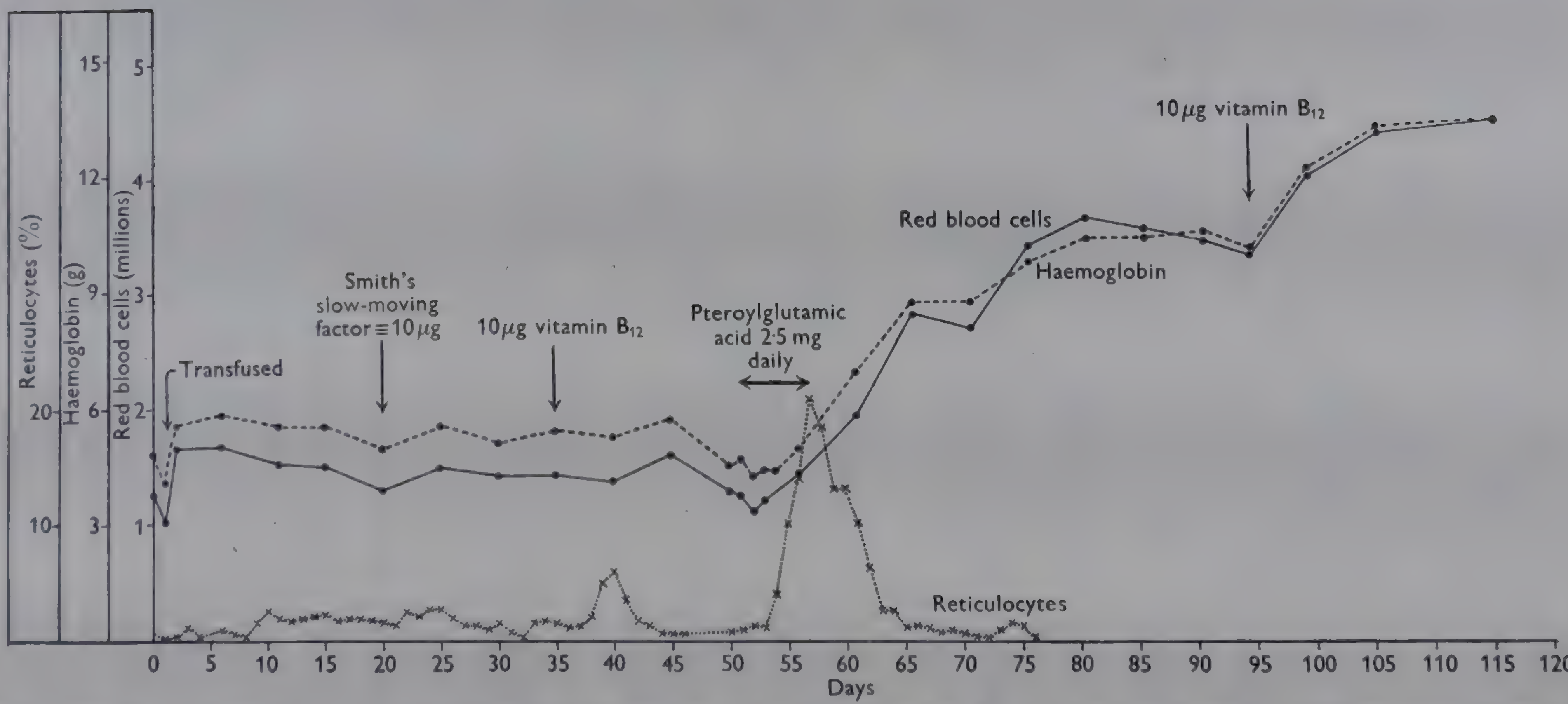


Fig. 2. Mr C., megaloblastic anaemia with syphilis. On the first two occasions 10 µg vitamin B_{12} or its equivalent had very little haemopoietic effect, although there was an excellent response to a total of 15 mg pteroylglutamic acid. When this response had ceased a similar dose of the same batch of vitamin B_{12} which had failed in the first instance now caused a satisfactory increase of red blood cells.

Reticulocytes reached a peak of 20·8 % on the 6th day, with rapid clinical and haematological improvement. On the 6th day the marrow was normoblastic.

	Days									
	0	5	10	15	20	25	30	35	40	44
Red blood cells (10^6/cu.mm)	1·24	1·42	1·93	2·80	2·67	3·39	3·62	3·53	3·43	3·30
Haemoglobin (g/100 ml.)	4·6	4·9	6·8	8·6	8·6	9·6	10·2	10·2	10·4	9·9
Packed cell volume (%)	14·5	17·0	—	32·0	30·0	35·0	37·0	—	—	—

After the effects of folic acid had waned, he received 10 μg of the same batch of vitamin B_{12} that had previously proved ineffective. This time the response was satisfactory.

	Days			
	0	5	11	21
Red blood cells (10^6/cu.mm)	3·30	3·98	4·35	4·45
Haemoglobin (g/100 ml.)	9·9	12·0	13·0	13·2

Normal blood values have since been maintained on vitamin B_{12} alone.

Although he had syphilis, this patient would probably have been regarded as suffering from true Addisonian pernicious anaemia but for the fact that he failed initially to respond to vitamin B_{12}.

The second possible example of dual deficiency (Mrs P., Fig. 3) is described under anaemias associated with idiopathic steatorrhoea (p. 308).

These cases recall the pigs described by Heinle, Welch & Pritchard (1948) which were so completely depleted of haemopoietic factors that folic acid failed unless liver extract was supplied; and liver extract failed unless small amounts of folic acid were supplied.

In some patients a small and delayed haemopoietic response has followed the administration of aureomycin (Lichtman, Ginsberg & Watson, 1950). Such responses —if they were indeed the effect of the antibiotics—may have arisen either from increased biosynthesis of citrovorum factor or vitamin B_{12} in the intestine, or from the elimination of bacterial sources of the (postulated) inhibitor.

It has long been a puzzle that some patients should get severe involvement of the central nervous system (or tongue) without anaemia. Girdwood (1951) suggests that such patients produce by intestinal biosynthesis enough folic acid to keep them free from anaemia, although they lack the vitamin B_{12} needed to protect the nervous system. Even if this hypothesis proves correct, we still need to determine why the nervous system remains undamaged in so many patients with severe and long-standing pernicious anaemia.

Depletion of vitamin B_{12} in pernicious anaemia is mainly due to loss of Castle's intrinsic factor. When doses of vitamin B_{12} as large as 3000 μg are given orally or *per rectum*, enough may be absorbed to produce a good haemopoietic response even though intrinsic factor is not supplied (Ungley, 1951). The absorption of small doses depends very much on the supply of Castle's intrinsic factor (see Ungley, 1950*a* part 2, Fig. 1). The mode of action of the latter is not known. Even when intestinal bacteria that absorb vitamin B_{12} are destroyed by antibiotics, intrinsic factor still potentiates the effect of small amounts of the vitamin administered orally (see Ungley, 1950*a*, part 4, Fig. 4 and Ungley, 1951–2). Moreover, normal gastric juice which has been heated sufficiently to destroy its intrinsic-factor activity may still retain the capacity to bind vitamin B_{12} (Spray, 1952). We are still exploring the possibility that interaction of vitamin B_{12} with intrinsic factor followed by enzymic splitting of the compound in the upper intestine leads to the release of a new compound which is more rapidly absorbed or utilized (Bethell, Swendseid, Miller & Cintron-Rivera, 1951; Ungley, 1951–2). It is unlikely, however, that the role of Castle's intrinsic factor is confined to absorption. Callender & Lajtha (1951*b*) have shown that, whereas neither free vitamin B_{12} nor unheated gastric juice is effective alone, the two together will

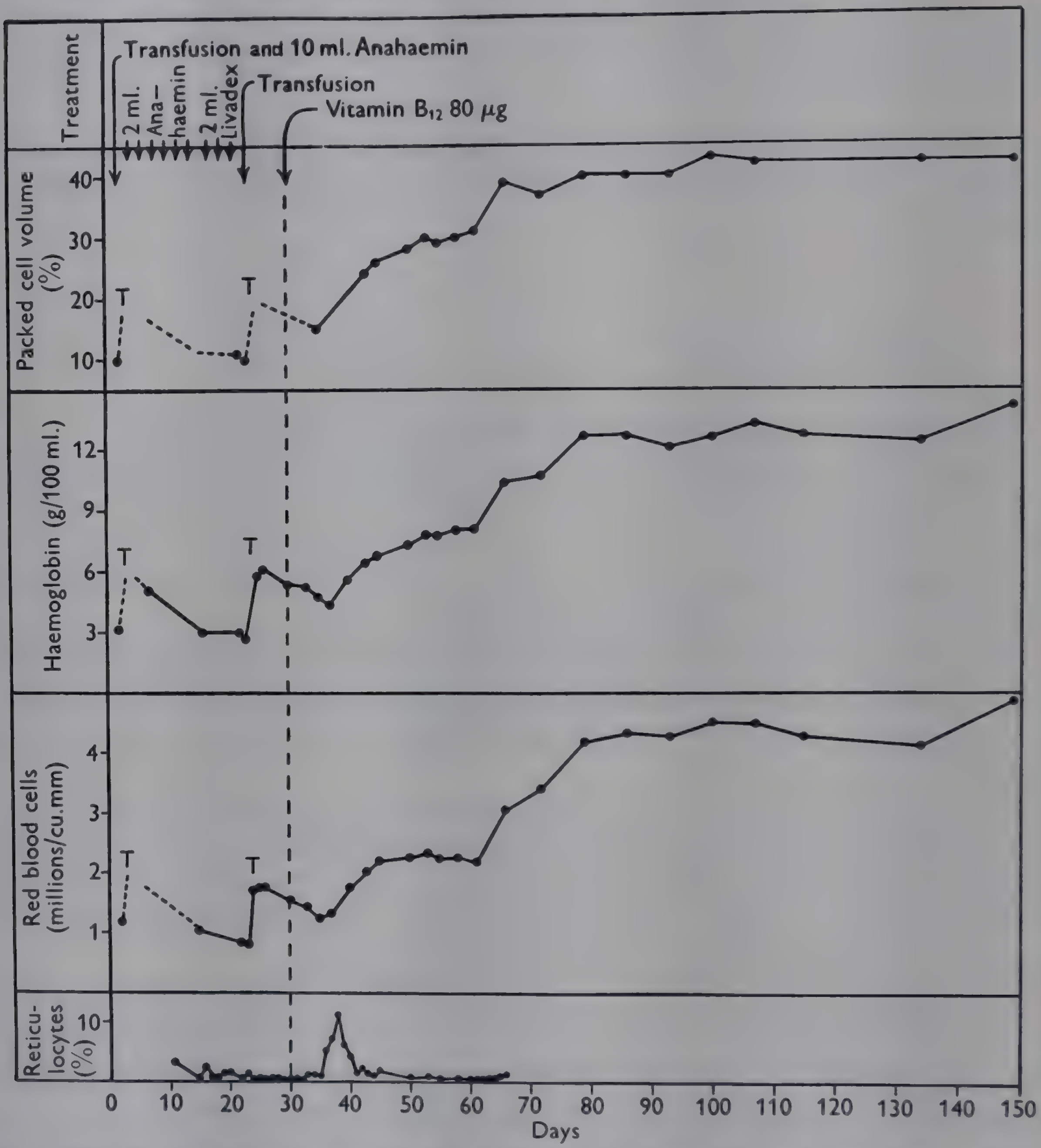


Fig. 3. Mrs P., megaloblastic anaemia with history of diarrhoea and poor diet. Vitamin B_{12} was ineffective at first, but effective later. In the interval she had received an injection of crude liver extract which had no haemopoietic effect, but may have supplied a missing nutrient needed for potentiating the action of vitamin B_{12} in this patient. T = transfusion.

change megaloblasts to normoblasts in marrow culture. The vitamin B_{12} in normal serum is likewise bound to a thermolabile substance and has a similar effect in marrow culture. Heating the serum or the mixture of normal gastric juice and vitamin B_{12} frees the vitamin and renders the material inactive in marrow culture. This suggests that

$$\text{intrinsic factor} + \text{extrinsic factor} = \text{haemopoietic factor}$$

as in Castle's original hypothesis.

Effects of vitamin B_{12}. The effects of parenterally administered vitamin B_{12} on symptoms referable to the anaemia, alimentary tract and the nervous system could be explained as direct, due to restoration of normal tissue metabolism, or as indirect, due to elimination of a toxic factor or inhibitor. Perhaps both actions are involved, the balance between nutrient and inhibitor governing the occurrence of relapse or remission.

To ascertain the relation between dose and response following a single injection, Ungley (1949*a*) spaced doses logarithmically: Fig. 1 in Ungley (1949*a*) shows some typical responses. Ten μg was just enough to produce, on average, a 'maximal' reticulocytosis and a rise of red blood cells which was 'optimal' according to standards for injectable liver extract. Individual variations were wide—especially for doses below 10 μg—but for groups of ten or more cases the deviation between observed and expected response was small. At least for doses ranging from 5 to 160 μg there was a constant relation between the logarithm of the dose and the mean increase of red blood cells in 15 days (see Ungley, 1949*a*, Fig. 5). A similar relationship exists between the logarithm of the dose and the mean increase of packed cell volume in 15 days. When the dose is increased to 320 μg this relationship no longer holds, so that 160 μg is probably somewhere near the top of the dose-response curve (Ungley & Campbell, to be published).

Other effects are: White blood cells and platelets usually increase to normal levels. The marrow changes from megaloblastic to normoblastic within about 72 h after a single injection. The effect lasts for days or weeks according to the size of the dose (Mollin & Dacie, 1950). Appetite returns and gain in weight follows. Soreness of the tongue is relieved in 4 or 5 days. After 2 or 3 weeks the papillae have usually regenerated and the tongue has its normal coating of fur.

Gastrointestinal symptoms usually vanish, but gastric atrophy persists and so does the achylia. Bilirubinaemia and the elimination of excessive amounts of urobilinogen cease (Concha, Etcheverry & Guzman, 1949). Changes in excretion of phenols and the elimination of an inhibitor from serum were mentioned earlier.

Here is an interesting example:

A woman had a very sore tongue with vivid red patches. The vitamin B_{12} content of her serum (Dr G. I. M. Ross) was 40 μμg/ml.—a low level. She had achlorhydria but no anaemia. A few days after a single injection of vitamin B_{12} she ate in comfort for the first time for 2 years, and within 2 weeks the tongue was entirely normal in appearance.

Liver extract versus vitamin B_{12}. In our experience vitamin B_{12} is in no way inferior to liver extract for initial therapy and maintenance in pernicious anaemia.

Low prothrombin values persisting after therapy with vitamin B_{12} (Owren, 1950) have not been encountered in our series (unpublished observations with Dr F. K. Herbert). The persistent macrocytosis reported by Larsen (1950) was not seen in Price-Jones curves of six cases treated solely with vitamin B_{12} for 2 or 3 years (Ungley, 1951–2).

Girdwood & Carmichael (1950) assayed liver extract microbiologically and clinically and found that the whole of the clinical activity could be accounted for on the basis of vitamin B_{12} (see also Girdwood, 1951).

Maintenance dose of vitamin B_{12}. Now that vitamin B_{12} is relatively cheap, enough should be given to cover wide individual variations and to maintain a reserve. The less frequent the injection, the more material is required to maintain remission. Some patients need more than others. A safe compromise is to give 100 μg every 2 weeks to patients with subacute combined degeneration, and 100 μg a month to those without neurological involvement. A few patients may need more frequent injections, and extra doses should always be given if intercurrent illness or infection supervenes.

Vitamin B_{12} and neurological manifestations. Vitamin B_{12} is as effective as liver extracts, crude or refined, in the treatment of subacute combined degeneration of the cord (Ungley, 1949*b*, Figs. 18 and 12). Paraesthesias diminish in extent and in severity, and may disappear. Difficulty in walking and ataxic gait almost always improve. Even patients who are hardly able to move their legs in bed may recover ability to walk. Incoordination of the extremities and Rombergism lessen or disappear.

Mental changes often clear up rapidly coincident with the relief of the anaemia. Some, which may be due to 'subacute combined degeneration of the brain', improve more gradually.

Optic atrophy is a less common but equally serious complication of pernicious anaemia.

Mr E. had achlorhydria, mild spinal cord involvement, early marrow changes and very little anaemia, but was virtually blind from optic atrophy. After treatment with vitamin B_{12} for 2 months the improvement in vision is already remarkable. He can read small newspaper headlines and walk through busy streets alone. Central scotomata are decreasing in size and density (Ungley & Maw, to be published).

The megaloblastic anaemias associated with idiopathic steatorrhoea

These form a mixed group. Some respond to vitamin B_{12}, others do not. Here are three examples from a larger series to be published later. In Mr McK. the response to 80 μg was about equal to the response expected from 5 μg in pernicious anaemia. In a former relapse the response to folic acid had also been slow. Red-cell counts seldom exceeded 4 millions/cu.mm with any form of therapy.

In a patient, Mr W., with steatorrhoea associated with thyrotoxicosis, the anaemia entirely failed to respond to vitamin B_{12}, whereas folic acid was effective. In this instance a toxic or haemolytic factor seemed to play a part; excessive haemolysis and rapid elimination of transfused cells from normal donors ceased about 2 weeks after the administration of folic acid.

The third case is a possible example of dual deficiency already referred to on p. 305 (Fig. 3). Mrs P., aged 71 years had abdominal pain and flatulence intermittently for

2 years. In a week she might eat bacon or an egg once, cabbage once or twice, little meat or fish, but plenty of potatoes, bread, sugar and tea. An attack of diarrhoea 2 months before admission was treated for 8 days with phthalylsulphathiazole. As the diarrhoea diminished, anaemia developed suddenly—so suddenly that melaena was wrongly suspected. Her condition improved slightly with iron and then deteriorated rapidly. When admitted she was thin, pale and drowsy. Red blood cells numbered 1,140,000/cu.mm, haemoglobin 2·9 g/100 ml., mean corpuscular volume 88 μ^3, mean corpuscular haemoglobin concentration 29 %, white blood cells 3300/cu.mm. The marrow was megaloblastic. There was achlorhydria. After transfusion, 10 ml. of 'refined' liver extract (Anahaemin, British Drug Houses Ltd.) were injected, then 2 ml. on alternate days. There was no response, and 12 days later red blood cells numbered 1,050,000/cu.mm. Three injections of 2 ml. 'crude' liver extract (Livadex, British Drug Houses Ltd.) were then given on alternate days. The total dose of vitamin B_{12} in the refined and crude liver extracts, kindly assayed by Dr W. F. J. Cuthbertson of Glaxo Laboratories, was not less than 70 μg. Again there was no response and after 5 days red blood cells had fallen to 830,000/cu.mm, haemoglobin was 3 g/100 ml., M.C.V. 132·5 μ^3, M.C.H. 35·5 $\mu\mu$g, mean corpuscular haemoglobin concentration 26·9 %, reticulocytes 0·6 %, white blood cells 4700/cu.mm.

When seen by us at this stage the patient was emaciated and pale, but cheerful. The bowels had been constipated, and the appetite good. The tongue was dry and smooth but never sore. The abdomen was distended but lax; the liver was enlarged half-way to the umbilicus; spleen was not palpable. Co-ordination of the limbs was normal, vibration sense unreliable. Idiopathic steatorrhoea was suspected because of the history of diarrhoea and the failure to respond to liver extract. Later a 2-day test on a diet containing 50 g fat showed 36 % fat in the dried faeces. Analysis of blood showed total proteins 6·15 g, albumin 3·72 g, globulin 2·43 g, calcium 8·0 mg and phosphorus 3·5 mg/100 ml., alkaline phosphatase 7·9 units, ascorbic acid absent, carotenoids absent, prothrombin 64 % of normal average, methaemalbumin absent, bilirubin 0·8 mg/100 ml. serum. A glucose-tolerance curve was within normal limits—fasting 105, maximum at $1\frac{1}{2}$ h 127 mg/100 ml. Radiography (performed when the patient was not having diarrhoea) showed apparent thickening of mucosal folds of the jejunum, but no segmentation of barium or delay in emptying the small intestine. After another transfusion and a satisfactory control period, during which reticulocytes did not exceed 1·4 %, 80 μg vitamin B_{12} were injected. Reticulocytes rose on the 6th day, reaching a peak of 11·2 % on the 8th day. The anaemia improved slowly, but normality was attained in 70 days without further therapy and without relaxing dietary restrictions.

	Days					
	0	5	10	15	20	25
Red blood cells (millions/cu.mm)	1·52	1·26	1·78	2·20	2·26	2·24
Haemoglobin (g/100 ml.)	5·3	4·6	5·6	6·8	7·3	7·7
Packed cell volume (%)	—	15·0	—	26·0	28·0	29·0

After about a year without therapy, and with the patient taking her ordinary diet, blood examination showed evidence of relapse. The decline in blood values was not arrested by the oral administration of vitamin B_{12} even when the dose was increased to 200 μg daily. There was no diarrhoea, but occasionally she passed two or three soft stools a day. The injection of 160 μg of vitamin B_{12} was followed by a rapid clinical and haematological improvement, red cells increasing from 2·69 to 4·55 millions/cu.mm in 15 days. For the last 5 months she has remained well without further injections. A second relapse occurred after 11 months without further injections. Normal blood values were restored following the injection of 50 μg vitamin B_{12}.

Megaloblastic anaemias of infancy

Vitamin C deficiency may be partly responsible for some of these anaemias—they are said to be much less common in America since a prominent manufacturer added ascorbic acid to his baby foods. As already mentioned, ascorbic acid facilitates the conversion of pteroylglutamic acid to citrovorum factor. May produced megaloblastic anaemia in monkeys by a diet deficient in ascorbic acid and in folic acid. In these scorbutic monkeys as little as 7·5 μg of citrovorum factor changed the marrow to normoblastic, whereas several milligrams of pteroylglutamic acid were needed to produce the same effect (May, Nelson, Lowe & Salmon, 1950; May, 1951).

In infants, however, the effective dose of citrovorum factor is much the same as that of pteroylglutamic acid. Moreover, some of the infants show no signs of vitamin C deficiency and some (unlike monkeys) respond to vitamin B_{12}, so that 'megaloblastic anaemias of infancy' are probably a mixed group too.

Macrocytic anaemias associated with cirrhosis of the liver

These anaemias are usually macrocytic and normoblastic. In a few cases where the marrow was megaloblastic, there is said to have been severe nutritional deficiency. One such patient responded excellently to vitamin B_{12} (Movitt, 1950).

Megaloblastic anaemia after total gastrectomy

One presumes that the mechanism of origin of these cases is the same as for Addisonian pernicious anaemia, which is also in a sense an 'agastric' anaemia. One of my patients is responding to 1000 μg vitamin B_{12} administered orally. In a case described by Conway & Conway (1951), however, the response to vitamin B_{12} was poor and incomplete, whereas folic acid quickly restored normal blood values.

Megaloblastic anaemias of pregnancy and the puerperium

In this country such anaemias usually fail to respond to vitamin B_{12} (see Fig. 1 in Ungley & Thompson, 1950 and Fig. 1 in Thompson & Ungley, 1951) and Castle's theory therefore cannot be invoked to explain them.

In Mrs A. vitamin B_{12} was ineffective, but the anaemia responded to folic acid (Ungley & Thompson, 1950, case 1). In Mrs M. E. H. the peripheral blood was rather microcytic and hypochromic, but the marrow was megaloblastic. Vitamin B_{12} failed, but there was a good response to folic acid. Iron was ineffective while the marrow was megaloblastic, but effective after folic acid had been supplied (Thompson & Ungley, 1951, p. 201).

A report on combined therapy with vitamin B_{12} and ascorbic acid (Holly, 1951) awaits confirmation. The basis for the haemopoietic efficacy of yeast extracts is uncertain and the existence of a 'Wills's factor' distinct from folic acid and the citrovorum factor has yet to be excluded. The anaemias respond to folic acid, but this does not necessarily imply that they are due to a deficiency of folic acid. The fallacy of such reasoning became evident after painful experiences with pteroylglutamic acid in pernicious anaemia.

I doubt whether megaloblastic anaemia of pregnancy is a nutritional disorder in the ordinary sense. Several of our patients had excellent diets, a good appetite, no vomiting or other alimentary disturbances, and no achlorhydria. In others, the diet, although not good, was at least no worse than that of other women who did not develop anaemia. Only in eleven out of twenty-seven in whom dietary histories were available could it be said that a deficient diet preceded the symptoms of anaemia (Thompson & Ungley, 1951).

One patient had a transient attack of fatty diarrhoea with definitely impaired fat absorption; this attack occurred between two pregnancies. In both these pregnancies megaloblastic anaemia developed but without diarrhoea or clinical evidence of steatorrhoea. Indeed diarrhoea of any kind was most unusual in our series. Much more evidence would be needed to incriminate malabsorption as a cause of megaloblastic anaemia in pregnancy and the puerperium.

The only constant aetiological feature is the relationship to pregnancy or the puerperium. The incidence is relatively higher in twin pregnancies and in multiparas, but the anaemia seldom recurs in subsequent pregnancies. An interesting possibility is that the development of anaemia depends on quantitative or qualitative differences in the output of sex hormones. The relationship between sex hormones and the erythrocyte count is well known. Sex differences between blood counts of male and female chicks (Taber, Davis & Domm, 1942–3) and rats (Vollmer, Gordon, Levenstein & Charipper, 1941) can be reversed by the administration of the hormone—oestrogen or androgen—appropriate to the opposite sex. The rapidity of growth of the genital tract of the chick (Hertz, 1948*b*) and monkey (Hertz, 1948*a*) in response to oestrogen varies with the amount of folic acid available. A normal supply of folic acid is adequate for a full androgen response in the male rat but not for a full oestrogen response in the female rat (Overbeek & Tausk, 1950). In the rat optimum lactation depends on the availability of folic acid (Cerecedo & Vinson, 1944).

Conceivably in man, too, there is an increased demand for folic acid as the uterus increases in size during pregnancy under the influence of oestrogen, and in susceptible persons this might lead to a deficiency of folic acid. But why are so few women susceptible? Do they produce excessive amounts of oestrogen? There is almost no information on this point. Day *et al.* (1949) record tests in one patient 5 days before delivery which showed an excretion of 4 mg of 17-ketosteroids, as compared with a normal range of 5·0–17·0 mg., in 24 h, 140 rat units of oestrogen in contrast to the normal 1000 or more rat units for the 9th month of pregnancy, and 15,300 i.u. of chorionic gonadotropins per 24 h, which is a high normal value. In one of our patients the excretion of ketosteroids, pregnanediol and total oestrogens at the 7th month of pregnancy was within normal limits (Thompson & Ungley, 1951).

The possibility remains that hormone output is qualitatively abnormal. Do these patients produce an abnormal steroid with properties antagonistic to folic acid? Chemically there is no similarity between steroid hormones and pteroylglutamic acid. It is of interest, however, that at least one sex hormone, dehydroisoandrosterone, has properties like folic acid for promoting the growth of certain micro-organisms (Gaines & Totter, 1950). The circulation of an abnormal steroid, if it exists, might explain the haemolytic manifestations seen in some cases of megaloblastic anaemia of pregnancy and the puerperium. In three patients red cells transfused from normal donors were rapidly destroyed until raw liver or folic acid was administered, when the rate of elimination became normal (Thompson & Ungley, 1951).

To summarize, then, there are three possible ways in which a deficiency of folic acid might arise in pregnancy and the puerperium:

(1) If the heavy but normal demands for folic acid at these times were not met from dietary sources because of dietary deficiency, vomiting or defective absorption. For reasons already given, this explanation is unlikely. It also fails to explain the rarity of recurrence in subsequent pregnancies.

(2) If the demand for folic acid were abnormally high, owing, say, to an excess of oestrogen. At present there is almost no evidence about demands for folic acid or about the level of oestrogen production in megaloblastic anaemia of pregnancy and the puerperium.

(3) If folic acid were rendered less available by the presence of an inhibitor or antagonist, arising perhaps from an abnormal variant of one of the steroid hormones. On such a hypothesis it would be easier to account for the haemolytic component in some cases of megaloblastic anaemia of pregnancy and the puerperium. Efforts to test these hypotheses continue.

Nutritional megaloblastic anaemias

These, attributed to dietary deficiency of either vitamin B_{12} or of the folic-acid group, are uncommon in temperate climates. Free hydrochloric acid is usually present in the gastric juice, as in the three cases mentioned below, or returns on treatment of the anaemia.

In two patients the anaemia responded to yeast extract or wheat germ. One was a widow aged 67 years, who had restricted her diet because of epileptic fits; and the other was a woman aged 39 with prolonged dietary deficiency and dysphagia of the Plummer-Vinson type (see cases 9 and 10, Figs. 2 and 3 in Ungley, 1933).

The third patient was a male epileptic aged 24, who had restricted his diet chiefly to tea and white bread and butter, consuming almost no meat, eggs, fresh green vegetables or fruit. The anaemia responded to parenteral liver therapy (see Fig. 1 in Ungley, 1938).

Tropical megaloblastic anaemias

In these, whether associated with pregnancy or not, dietary deficiency is the rule, and vitamin B_{12} is often effective (Patel, 1948; Patel & Kocher, 1950; Das Gupta (personal communication), 1951; Chaudhuri, 1951).

Megaloblastic fish-tapeworm anaemia

von Bonsdorff and his colleagues in Helsinki have done much to advance our knowledge in this field (von Bonsdorff, 1948; von Bonsdorff & Gordin, 1951, 1952; Björkenheim, 1951). Infestation with *Diphyllobothrium latum* is common in country districts in Finland, but very few of those infested develop megaloblastic anaemia. In those who do, the worm is situated high up in the small intestine. The clinical picture resembles Addisonian pernicious anaemia even to the incidence of neurological phenomena, but there is usually free HCl in the gastric juice. Symptoms can be relieved either by expelling the worm or by giving vitamin B_{12} parenterally with the worm still in situ.

Many attempts have been made to isolate a toxin from the worm. Now it appears that far from being toxic, the dried worm is curative, being a rich source of vitamin B_{12}! Presumably the living worm in the intestine takes up vitamin B_{12} of which the host is thus deprived. Dietary deficiency and depression of gastric secretion, if present, are of secondary importance. Whether or not an inhibitor is present in the serum of the patient has yet to be determined.

Conclusions

In the management of patients suspected of having megaloblastic anaemia it is essential to examine the marrow before treatment obscures the picture. The blood findings alone may be misleading, for not all macrocytic anaemias are megaloblastic and not all megaloblastic anaemias are macrocytic.

Controlled therapeutic tests are important for diagnosis and prognosis. If vitamin B_{12} fails, the effect of some form of folic acid, e.g. pteroylglutamic acid or citrovorum factor, should be observed. Even if vitamin B_{12} is effective, the anaemia may not be Addisonian. If free hydrochloric acid is found in the gastric juice, other tests such as fat balance or radiology of the intestine may be required. These may reveal some underlying disorder such as intestinal stenosis, gastrocolic fistula or idiopathic steatorrhoea.

In Addisonian pernicious anaemia vitamin B_{12} is as effective as liver extract even for the neurological manifestations. Certain non-Addisonian megaloblastic anaemias also respond to vitamin B_{12}, but there are others that do not. In these the missing nutrient may be folic acid or possibly Wills's factor. In rare cases lack of one nutrient seems to inhibit the response from another, as though dual deficiencies were present.

In the past 100 years theories about megaloblastic anaemias have swung from 'nutritional' to 'toxic' and back to 'nutritional' again. Now the opposing theories can be united in a single working hypothesis which postulates an interplay between nutritional and toxic or inhibiting factors. The future lies in the integration of work in many fields, and it is a pleasure to thank all those who are collaborating to this end.

I am particularly indebted to Drs G. I. M. Ross, Freda K. Herbert, A. L. Latner, R. B. Thompson, E. V. Cox, Mrs Laureen Raine and Mr W. Elsbury, and to the medical, technical and secretarial staff of my clinic and laboratory. Also to Drs E. Lester Smith and W. F. J. Cuthbertson of Glaxo Laboratories, Ltd., for help and advice, and for supplies of vitamin B_{12}.

The work was aided by grants from the Medical Research Council and Lady Tata Memorial Trust.

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The Relationships between Vitamin B_{12}, Folic Acid and Folinic Acid

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The interrelationships between the various substances used for the treatment of the megaloblastic anaemias are complex and little understood. The vast amount of experimental work carried out in recent years has given us so many papers that it is almost impossible to keep track of them, but the jig-saw is as yet incomplete. In the short time available it is not possible to deal fully even with one aspect of the problems, which have been considered in more detail in a recent review (Girdwood, 1952*a*); to-day I shall refer briefly to some of the reported work and add data derived from a few metabolic studies carried out on patients.

Chemical interrelationships

Dr Lester Smith has discussed the formula of vitamin B_{12} and related forms (Smith, 1952). As is known, the name folic acid is usually given to synthetic pteroylglutamic acid, although it is not certain that the two are identical (Robinson, 1951). Pteroylglutamic acid is not related chemically to vitamin B_{12}. It has long been considered that the chief form of folic acid present in foodstuffs is that found in yeast, pteroylhexaglutamylglutamic acid, a γ-linked peptide which has not been synthesized. Satisfactory experiments cannot be carried out with this substance because of the presence of 'conjugase inhibitors' in the yeast concentrates used as a source of this natural folic-acid conjugate.

Synthetic conjugates of pteroylglutamic acid are pteroyldiglutamic acid and pteroyltriglutamic acid (Diopterin and Teropterin, Lederle Laboratories Inc.). As far as is known, these do not occur in food and, in fact, the former does not occur naturally.

Folinic acid (Bond, Bardos, Sibley & Shive, 1949) appears to be the same substance as the citrovorum factor (Sauberlich & Baumann, 1948), so called because it will support the growth of the streptococcus *Leuconostoc citrovorum*. The chemical structure of this substance is reported to be 5-formyl-5:6:7:8-tetrahydropteroylglutamic acid. We do not know much about conjugate forms of folinic acid but, as will be explained later, it is likely that in certain tissues and organs the activity that has been attributed to folic acid or its conjugates is due at least in part to folinic acid or its conjugates.

Microbiological interrelationships and quantitative estimation of these haemopoietic factors

Under suitable assay conditions, vitamin B_{12} is a growth factor for *Lactobacillus leichmannii*, *Lb. lactis* Dorner, *Euglena gracilis* and *Bacterium coli*.

I shall not consider further the difficulties involved in carrying out the assays, as this is dealt with by Ford (1952).

Pteroylglutamic acid is usually estimated by its growth-promoting effect upon *Streptococcus faecalis* R or *Lb. casei*. Pteroyldiglutamic acid will not support the growth of either of these organisms, whereas pteroyltriglutamic acid supports the growth of *Lb. casei* but not that of *Strep. faecalis* unless present in high concentration. Thymine and thymidine (thymine deoxyriboside) can replace pteroylglutamic acid for the growth of *Lb. casei* and *Strep. faecalis*.

The citrovorum factor is measured quantitatively by virtue of its growth-promoting effect upon *Leuc. citrovorum*. However, it will also support the growth of *Lb. casei* and *Strep. faecalis* and the true folic-acid content of substances being tested is arrived at by a differential assay method, using both *Lb. citrovorum* and *Strep. faecalis* as test organisms (Swendseid, Bethell & Ackermann, 1951). Under the conditions of my own differential assays, citrovorum factor has about 76 % of the growth-promoting effect of pteroylglutamic acid for *Strep. faecalis*.

Thymidine can diminish the requirements of *Leuc. citrovorum* for folinic acid, but folic acid is inactive for *Leuc. citrovorum* unless present in very high concentration.

Other complications are many, and time does not permit me to mention them. I have found the folinic-acid assay to be far the most consistent and satisfactory. With the other assays difficulties are very frequent.

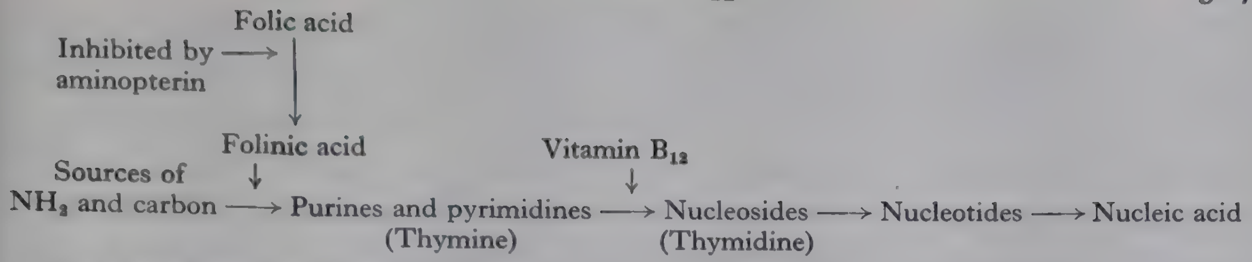
Stokes (1944) could not demonstrate folic acid in organisms grown in a medium containing thymine in place of folic acid and suggested that folic acid functions as a coenzyme for the enzyme system responsible for the synthesis of thymine, the latter being used by the bacteria to form nucleic acid:

$$\begin{tikzcd}[column sep=small] & \text{Folic acid} \arrow[d] & & \\ \text{Precursors} \arrow[rr] & {} & \text{Thymine} \arrow[r] & \text{Nucleic acid} \end{tikzcd}$$

Wright, Skeggs & Huff (1948) suggested that vitamin B_{12} functions as a coenzyme for the conversion of thymine to thymidine:

$$\begin{tikzcd}[column sep=small] & \text{Folic acid} \arrow[d] & & \text{Vitamin } B_{12} \arrow[d] & & \\ \text{Precursors} \arrow[rr] & {} & \text{Thymine} \arrow[rr] & {} & \text{Thymidine} \arrow[r] & \text{Nucleic acid} \end{tikzcd}$$

Nichol & Welch (1950) produced evidence to support the view that folinic acid is the 'biologically active' form of folic acid and hence we have one theory of the interrelationships between these factors as follows:



This has been elaborated by Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins & Seaman (1950), who claim that thymine is effective in large doses in pernicious anaemia even after folic acid has had no effect. Hausmann (1949) has reported a response to thymidine in large dosage in pernicious anaemia, but I have been unable to obtain sufficient of this substance to carry out clinical experiments.

Interrelationships in animal experiments

Megaloblastic anaemia can be produced in certain animals, notably pigs, by dietetic means, by the use of folic-acid antagonists, or by both. It is considered that by such means the animals may be made deficient in both vitamin B_{12} and folic acid. Responses have occurred following treatment with pteroylglutamic acid, pteroyldiglutamic acid, pteroylhexaglutamylglutamic acid (Heinle, Welch, George, Epstein & Pritchard, 1947, Cartwright; Tatting, Ashenbrucker & Wintrobe, 1949). Responses to liver injections, vitamin B_{12}, proteolysed liver, marmite, deoxyribosenucleic acid, ribosenucleic acid, thymine and histidine have been poor (Cartwright, Palmer, Tatting, Ashenbrucker & Wintrobe, 1950). There is one paper describing pigs that appeared to become so depleted of vitamin B_{12} and folic acid that folic acid failed to give a response until vitamin B_{12} injections (liver injections) were given and, conversely, liver injections failed unless folic acid was given (Heinle, Welch & Pritchard, 1948). In general, the experiments indicate the importance of supplying both vitamin B_{12} and folic acid to prevent megaloblastic anaemia in the pig. Protein deficiency increases the need for folic acid.

In considering megaloblastic anaemia in the monkey, we are faced with the problem of Wills's factor. As is well known, Wills studied nutritional megaloblastic anaemia in man in India, and in monkeys (Wills & Stewart, 1935; Wills, Clutterbuck & Evans, 1937; Wills & Evans, 1938). Anahaemin (British Drug Houses Ltd.), which was active in pernicious anaemia, was inactive in nutritional megaloblastic anaemia. Campolon (Bayer Products Ltd.) was active in pernicious anaemia and, when separation with ammonium sulphate was carried out, both the soluble and insoluble fractions were active in this disorder. However, only the soluble fraction was active in nutritional megaloblastic anaemia in man and in the monkey. Strauss & Castle (1932) state that the autolysed yeast preparation, marmite, which does not contain vitamin B_{12}, is active by mouth in pernicious anaemia only if given with gastric juice. However, marmite was active in nutritional megaloblastic anaemia. The main problem is whether Wills's factor is folic acid, folinic acid, or some other unindentified substance. The monkeys received 5 g of marmite daily (Wills & Stewart, 1935) an amount that is said to contain 0·3 mg of growth factors for *Strep. faecalis* (as pteroylglutamic acid). However, Day & Totter (1948) have shown that this is more than sufficient to protect the animals against anaemia. The normal daily intake of folic or folinic acid required to prevent nutritional megaloblastic anaemia in man is uncertain; it is probably less than 100 μg/day; the minimal amount required to treat a patient with pernicious anaemia is quite another matter. It is known that the content of folic and folinic acid in present-day liver extracts is very small (usually less than 1·5 μg/ml.), but what we really would need to know is the content of these factors in Campolon in 1931–7 when Wills was doing her researches, and attempts are being made at present to obtain such material. The action of marmite in Wills's experiments could be explained on its content of growth factors for *Strep. faecalis*, though why marmite is not more effective in pernicious anaemia or why gastric juice potentiates its action is not fully understood. Perhaps the folic or folinic acid of marmite is present in a conjugate form from which it is released or made available by gastric juice, or perhaps gastric juice neutralizes inhibitors. Full understanding of the significance of separation by ammonium sulphate would appear to necessitate a knowledge of the effect of such separation not only upon folic acid and folinic acid but also upon their conjugates.

To complicate the experiments with monkeys further, it is now known that in the monkey placed on a deficient diet, megaloblastic anaemia develops more rapidly if there is ascorbic-acid deficiency. When it does develop, folic acid is rapidly effective in treatment, ascorbic acid is more slowly effective, vitamin B_{12} is ineffective, and vitamin B_{12} together with ascorbic acid gives a prompt response (May, Nelson, Lowe & Salmon, 1950). In infants, too, megaloblastic anaemia due to folic-acid deficiency occurs more rapidly if there is ascorbic-acid deficiency.

I shall not deal at all with the animal protein factor or with the effects of antibiotics, as these are being discussed separately (Coates, 1952; Cuthbertson, 1952).

Other metabolic interrelationships

It is now clear that folic acid and vitamin B_{12} take part in many processes concerned with the utilization of proteins not only for haemopoiesis but also for growth and development. For example, it has been shown in chicks that folic acid is involved in the conversion of choline to betaine and in transmethylation from betaine to methionine (Dinning, Keith & Day, 1951). Vitamin B_{12} also functions in methionine formation (Oginsky, 1950). Again, glycine toxicity in young chicks can be overcome by vitamin B_{12} and to a lesser extent by folic acid, but the two together are more effective than either alone (Machlin, Denton & Bird, 1951). These and other interrelationships could well be the subject of a symposium in themselves.

Bone-marrow cultures

In bone-marrow cultures, Rusznyák, Löwinger & Lajtha (1947) showed that the serum contained a factor that inhibited the conversion of megaloblasts to normoblasts. Folic acid and folinic acid caused such conversion, whereas vitamin B_{12} was ineffective except when mixed with a source of intrinsic factor (Callender & Lajtha, 1951). Lajtha (1950) has suggested that folic acid (or folinic acid) acts directly on the red-cell precursors, but that this activity is inhibited normally by a factor in the serum. This inhibitory factor is counteracted by vitamin B_{12} after the latter has been converted to an active form in the body. On the other hand, Horrigan, Jarrold & Vilter (1951), who instilled folic acid, folinic acid and vitamin B_{12} directly into the bone marrow of patients, reported that, whereas vitamin B_{12} would convert megaloblastic cells to the normoblastic state, folic acid and folinic acid were not effective. This last approach requires further study.

Metabolic and therapeutic studies in man

At an early stage in the investigations of the action of folic acid in the megaloblastic anaemias, Bethell, Meyers, Andrews, Swendseid, Bird & Brown (1947) suggested that an important function of the liver factor (vitamin B_{12}) is to liberate folic acid from its conjugates, and that this cannot occur in pernicious anaemia. This conclusion was based upon measurements of urinary excretion of folic acid in pernicious-anaemia patients and controls after the administration of the naturally occurring conjugate, pteroylhexaglutamylglutamic acid. Unfortunately difficulties arose in confirming this work because it was found that the sources of the natural conjugate contained 'conjugase inhibitors' that complicated the experiments without altogether invalidating them. In support of this theory was the fact that all forms of megaloblastic anaemia responded in the first instance to folic-acid therapy. It is now known that in some patients treated for a period of years with folic acid, haematological relapse may occur. I should like to describe briefly such a patient, part of whose story has already been published (Girdwood, 1952*a*).

The patient was a male aged 23 years, suffering from the sprue syndrome with 68 % absorption of fat; the test meal showed histamine-fast achlorhydria. He had a markedly megaloblastic marrow although he had been taking folic acid by mouth in a dosage of 5 mg daily for more than 2 years. As will be seen from Fig. 1 there was then no response to 15 mg folic acid daily intramuscularly, but 90 mg daily converted the marrow to the normoblastic state and led to a rise in the red cells. This improvement was not maintained with 90 mg of folic acid intramuscularly weekly, and the patient was readmitted to hospital in November 1951, with pneumonia. The marrow was again megaloblastic.

The lung infection did not respond well to sulphonamide therapy. I was anxious to try the effect of vitamin B_{12} on the blood picture of this patient who was not in the ward to which I am attached. Unfortunately on the very day that this treatment was given the doctor who was responsible for attending to the patient was himself taken ill and penicillin was given to the sprue patient without my prior knowledge. This perhaps complicates matters since there have been reports from Africa of responses to penicillin in megaloblastic anaemia, but in any event it can be said that the blood picture was restored rapidly to normal although the haemopoietic tissues had become refractory to very large doses of folic acid.

I imagine that the most likely explanation of this response is that the patient was unable to absorb vitamin B_{12} and that folic acid could not be effective in its absence. It might be suggested that folinic acid would have been effective and that perhaps the function of vitamin B_{12} is to play some part in the conversion of folic acid to folinic acid. However, Jarrold, Horrigan, Thompson & Vilter (1951) have reported on a patient with pernicious anaemia who became refractory to folic acid. There was no response to Lederle's synthetic preparation of citrovorum factor, but vitamin B_{12} was effective.

In support of the theory that vitamin B_{12} releases folic acid from its conjugates, it has been reported (Suárez, Welch, Heinle, Suárez & Nelson, 1946; Anonymous, 1947) that liver-extract injections give rise to increased urinary excretion of folic acid in pernicious anaemia and sprue. As will be seen in Fig. 2, that has not been our experience.

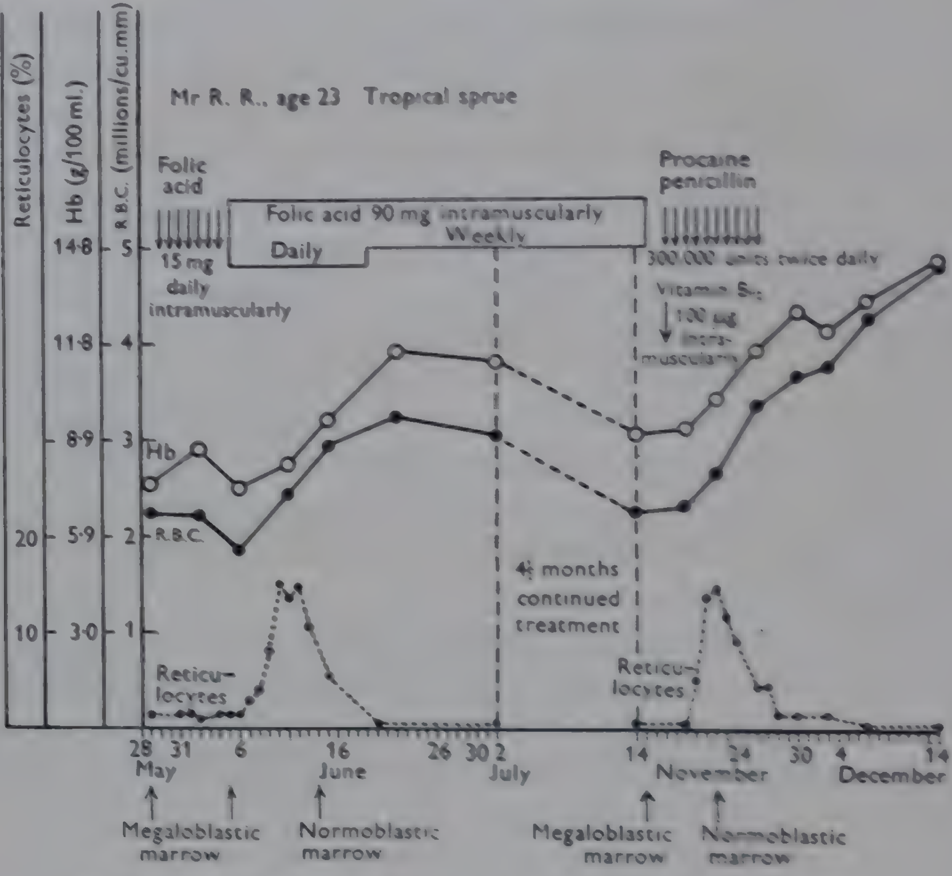


Fig. 1. Megaloblastic anaemia refractory to folic-acid therapy.

If synthetic folic-acid conjugates are given by mouth or by injection to patients with pernicious anaemia, there is a haemopoietic response and excretion of pteroylglutamic acid. Table 1 shows the urinary excretion of pteroylglutamic acid in eight patients treated in this way—in only two patients given the dose indicated for several days was there no haemopoietic response. All that this experiment shows, however, is that the pernicious-anaemia patient can convert these particular synthetic forms to 'free' folic acid. For comparison, the average excretion of folic acid in the urine of eight pernicious-anaemia patients given 5 mg pteroylglutamic acid intramuscularly was 1·05 mg.

Finally, I want to refer briefly to a few of the metabolic mysteries of the megaloblastic anaemias. Mollin & Ross (1951), using *Euglena gracilis* as test organism, have found that the urinary and serum values for vitamin B_{12} are lower in pernicious-anaemia patients than in controls. On the other hand, using *Lb. leichmannii* (and correcting for deoxyribosides) we have found similar amounts of vitamin B_{12} in the urine in pernicious-anaemia patients and in normal persons. One possibility is that the *Lb. leichmannii* assay estimates as yet unidentified substances other than vitamin B_{12}

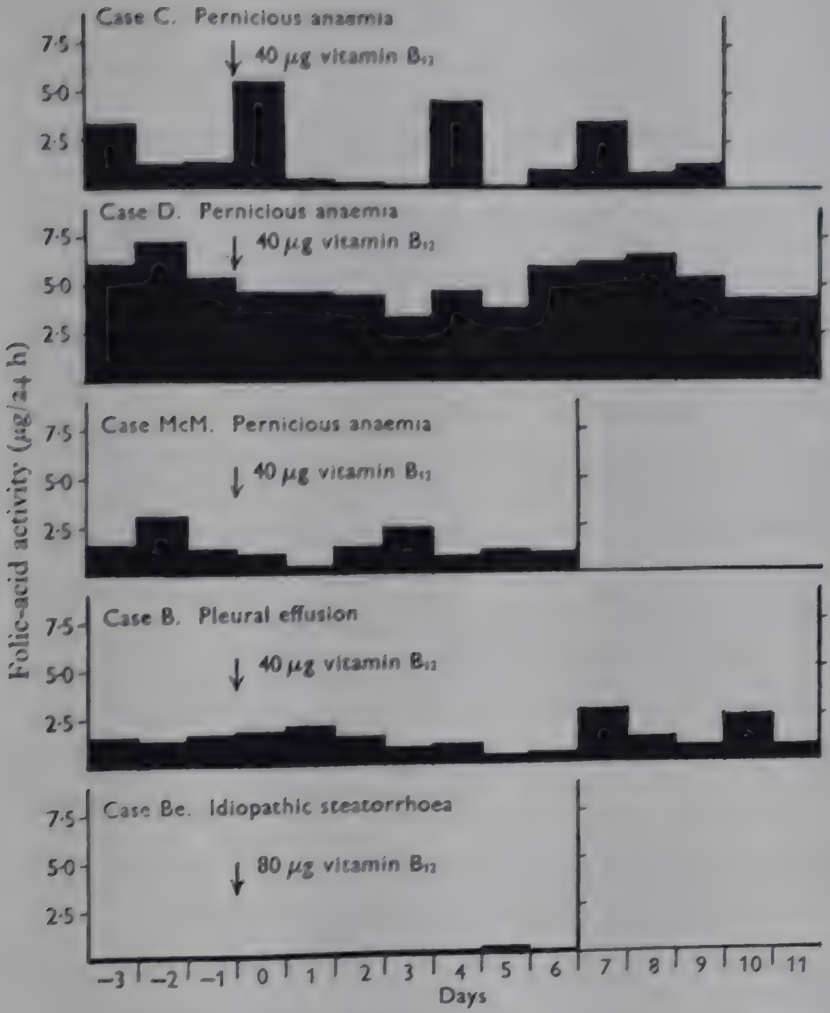


Fig. 2. Effect of vitamin B_{12} given intramuscularly on total folic-acid activity of urine.

Table 1. *Urinary output of pteroylglutamic acid following administration of its synthetic conjugates in pernicious anaemia*

Case	Drug	Dose (mg)	Route*	Folic-acid activity of urine: Before treatment (µg/24 h)	Folic-acid activity of urine: After treatment (mg/24h)	Remarks
McL.	Pteroyltriglutamic acid	10	IM	1·6	1·880	—
C.	Pteroyltriglutamic acid	10	Oral	2·8	1·440	—
R.	Pteroyltriglutamic acid	2·5	Oral	3·2	0·036	—
McG.	Pteroyltriglutamic acid	2·5	Oral	1·5	0·006	—
P.	Pteroyldiglutamic acid	5	IM	1·1	0·280	No haematological response with 18 days' treatment. Response to 5 mg PGA, IM, daily
L.	Pteroyldiglutamic acid	5	Oral	1·3	0·330	—
H.	Pteroyldiglutamic acid	2·5	IM	0·3	0·370	No response
S.	Pteroyldiglutamic acid	2·5	IM	1·6	0·012	—

* IM = intramuscular.

that are destroyed by alkaline hydrolysis. If so, it is surprising that there is not a greater difference between the values for patients and controls. Recent results for the urinary content of haemopoietic factors are given in Table 2.

Table 2. *Urinary content of vitamin B_{12}, folic acid and folinic acid (citrovorum factor)*

Case	Diagnosis	Vitamin B_{12} content (mμg/24 h)	Folic-acid activity (μg/24 h)	Folinic-acid content (μg/24 h)	True folic-acid content (μg/24 h)
A.	Megaloblastic anaemia of pregnancy	Nil	1·60	1·92	0·14
G.	Pernicious anaemia	121·5	2·30	0·80	1·69
S.	Pernicious anaemia	108·1	1·61	0·45	1·27
H.	Pernicious anaemia	102·0	0·33	0·20	0·18
Ar.	Pernicious anaemia	71·7	2·90	0·41	2·59
Gi.	Pernicious anaemia	45·5	8·70	2·90	6·50
B.	Pernicious anaemia	36·5	0·97	0·34	0·71
W.	Pernicious anaemia	35·0	1·80	1·00	1·04
R.	Pernicious anaemia	32·4	2·12	0·92	1·42
S.	Pernicious anaemia	21·4	2·20	0·62	1·73
P.	Pernicious anaemia	Nil	2·40	0·73	1·84
T.	Pernicious anaemia	Nil	2·20	1·35	1·15
M.	Normal	198·6	2·03	0·52	1·64
Ro.	Normal	120·5	4·47	1·12	3·62
D.	Normal	Negligible	0·61	0·45	0·27
K.	Normal	Nil	3·98	0·76	3·47

Table 3. *Urinary excretion of citrovorum factor following its administration by mouth or by injection*

		Treatment with Leucovorin*		Urinary excretion in 24h after treatment		
				Folic-acid activity†	Citrovorum-factor activity	
Case	Diagnosis	Dose (mg)	Route‡	(*Strep. faecalis* assay) (μg/24 h)	*Strep. faecalis* assay (μg/24 h)	*Lb. citrovorum* assay (μg/24 h)
F.	Pernicious anaemia	3	Oral	7·4	—	0·199
S.	Pernicious anaemia	3	Oral	18·7	30·8	30·4
		6	Oral	26·8	31·0	41·5
G.	Pernicious anaemia	12	Oral	155·0	103·3	135·9
Si.	Pernicious anaemia	12	Oral	28·7	39·7	30·6
Sn.	Pernicious anaemia	3	IM	202·0	—	302·0
W.	Pernicious anaemia	9	IM	1100·2	—	1560·4
C.	Pernicious anaemia	12	IM	1850·5	—	1870·3
Sh.	Pernicious anaemia	12	IM	583·5	—	791·5
McF.	Pernicious anaemia	12	IM	352·4	—	480·2
A.	Megaloblastic anaemia of pregnancy	12	Oral	99·5	120·3	100·1
W.	Normal	12	Oral	99·5	134·8	70·6
McL.	Normal	12	Oral	99·6	133·6	76·7
Ro.	Normal	12	IM	1227·2	1761·0	1827·4
Re	Normal	12	IM	957·3	1264·4	1110·0

* Lederle Laboratories Inc. † Due largely to citrovorum factor. ‡ IM = intramuscular.

Pernicious-anaemia patients will respond to 3–12 mg of folinic acid whether it be given by mouth or by injection, the smaller doses being administered daily (Davidson & Girdwood, 1951 *a*, *b*). However, as will be seen from Table 3, the urinary output of folinic acid is much less when it is given by mouth, although it is haemopoietically active by both routes and is not excreted as folic acid. If this is merely a matter of poor absorption by the alimentary route, it is surprising that oral treatment is satisfactory. In contrast, if folic acid is given in comparable dosage, the urinary output of folic acid is similar whether it be given by injection or by mouth, and only a small proportion (0·1 % or less) is excreted as the citrovorum factor.

The livers of two non-anaemic control patients examined at autopsy contained 162·3 and 523·7 μg/100 g respectively of citrovorum factor, but no folic acid. At autopsy, the liver of a pernicious-anaemia patient who died the day after having been given 100 μg. vitamin B_{12}, contained 78·9 μg citrovorum factor and 61·6 μg folic acid per 100 g (Girdwood, 1952*b*). This should only be taken as an indication for further investigation along similar lines.

Holly (1951), acting on the suggestion of May *et al.* (1950) that ascorbic-acid deficiency may interfere with the conversion of folic acid to folinic acid, has treated patients with megaloblastic anaemia of pregnancy with vitamin B_{12} and ascorbic acid. In three such patients, vitamin B_{12} and ascorbic acid separately were ineffective, whereas the two together gave a haematological remission.

These results, if confirmed, suggest that, as in the megaloblastic anaemias of infants and of monkeys, ascorbic-acid deficiency plays a part in the causation of at least some cases of megaloblastic anaemia of pregnancy.

Conclusions

All that we can conclude at present is that vitamin B_{12} and folic acid both appear to be required for normoblastic blood formation. It is probable that folic acid is normally converted in the body to folinic acid, but this latter substance is not the end of the metabolic chain. There is some indirect evidence for the presence of inhibitory factors (Lajtha, 1950; Ungley, 1951), but their existence is still problematical. Ascorbic-acid deficiency appears to be related to the development of megaloblastic anaemias, although this form of anaemia is not found in scurvy itself. The existence of other factors is possible. We have seen that Wills's factor has not yet been satisfactorily explained. Moreover, in certain forms of anaemia where the marrow is frequently megaloblastic (e.g. in sprue) there are some cases where the marrow is not truly megaloblastic but contains cells intermediate between megaloblasts and normoblasts. This type of anaemia frequently fails to respond to vitamin B_{12} or folic acid, possibly because some other unknown factor is lacking.

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The Microbiological Assay of Vitamin B_{12}

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The literature on the microbiological assay of vitamin B_{12} is large and growing. Those familiar with microbiological techniques realize their limitations, but there is a danger that the apparent ease with which the tests can be carried out may encourage uncritical acceptance of the spate of results now appearing. An object of this paper is to show some of the difficulties that may be involved in the assay of vitamin B_{12}.

To begin with there are the ever-present fundamental problems of specificity of test and completeness of extraction. There is next the problem of the total extraction and measurement as vitamin B_{12} of the series of related substances, all active microbiologically, that may be present in natural products, and that are distinguished from vitamin B_{12} only by the replacement of the cyano-group by a variety of complex-bound anions (Brink, Kuehl & Folkers, 1950; Kaczka, Wolf, Kuehl & Folkers, 1950; Smith, 1948; Smith, Fantes, Ball, Ireland, Waller, Emery, Anslow & Walker, 1951; Pierce, Page, Stokstad & Jukes, 1949; Wijmenga, Veer & Lens, 1950).

A further problem arises from the presence in some natural materials of other substances also related to vitamin B_{12} but differently active for different assay organisms. They are not in the strict sense cobalamins and cannot be converted to vitamin B_{12} by treatment with cyanide (Coates, Ford, Harrison, Kon, Porter, Cuthbertson & Pegler, 1951; Coates, Ford, Harrison, Kon & Porter, 1952; Ford, Kon & Porter, 1951; Ford & Porter, 1952; Pfiffner, Calkins, Peterson, Bird, McGlohon & Stipek, 1951; Wijmenga, 1951).

Finally, vitamin B_{12} may also be present in some natural materials in a microbiologically inactive, 'bound' form (Bird & Hoevet, 1951; Smith, 1950–1; Meyer, Eppstein, Bethell & Hall, 1950; Shaw, 1950; Ternberg & Eakin, 1949).

It will be helpful to begin with a brief summary of some of the more commonly used microbiological methods.

The lactobacilli as test organisms

Under specified conditions, several of the lactic-acid bacteria require vitamin B_{12}. Of these, strains of *Lactobacillus leichmannii* and *Lb. lactis* are widely used to assay the vitamin. The requirement for vitamin B_{12} is not specific, in the sense that in the nutrition of these organisms certain nucleic-acid derivatives or deoxyribonucleic acid can replace, or stimulate the response to, vitamin B_{12}. The effects of these substances can, however, be minimized and often altogether eliminated, particularly in tube assays, by the use of appropriate techniques (see review by Snell & Wright, 1950 and Skeggs, Nepple, Valentik, Huff & Wright, 1950).

Vitamin B_{12b}, one of the cobalamins present in natural materials, is readily inactivated for *Lb. leichmannii* on heating with the assay medium (cf. Soars & Hendlin, 1951) and may thus be lost in the process of measurement. Vitamin B_{12b} is readily converted into vitamin B_{12} by treatment with cyanide (Brink *et al.* 1950; Ellis, Petrow, Beaven, Holiday & Johnson, 1950; Veer, Edelhausen, Wijmenga & Lens, 1950) and the presence of minute amounts of cyanide prevents inactivation of vitamin B_{12b} on heating with the assay medium, apparently by converting it to the stable vitamin B_{12} (Cooperman, Drucker & Tabenkin, 1951; Soars & Hendlin, 1951). Cooperman *et al.* found with some products that only with media containing cyanide did *Lb. lactis* assays agree with those by *Lb. leichmannii*.

Assays with Bacterium coli

A mutant of *Bact. coli* requiring vitamin B_{12} is now increasingly used in a plate method perfected by workers at the Glaxo Laboratories (Bessell, Harrison & Lees, 1950; Cuthbertson, Pegler & Lloyd, 1951; Harrison, Lees & Wood, 1951). The method is speedy and simple in operation and allows large numbers of assays to be carried out on a routine basis. It is subject to interference by methionine and to a lesser extent by other substances, but in general such interference is readily detected from the appearance of the zones of exhibition.

Burkholder (1951) has described the use of this organism in a tube assay and reported good agreement between assays of a number of substances with it and with *Euglena gracilis*. This tube-assay technique is more sensitive than the plate method, which is best suited to the assay of higher-potency materials and extracts.

Assays with Euglena gracilis

The high sensitivity and apparent specificity of *Euglena gracilis* as compared with other assay organisms (Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin & Jukes, 1949; Picken & Bauriedel, 1950) has recommended its use with low-potency materials. Robbins, Hervey & Stebbins (1950, 1951) have used it to measure the vitamin B_{12} content of a wide range of natural materials, Ross (1950) for body fluids and liver extracts, but Picken & Bauriedel (1950) found the method satisfactory only for the assay of relatively pure preparations. They described a method of using the organism, together with *Lb. leichmannii*, in comparative bioautographic studies of vitamin B_{12} and related growth factors.

Experience at Shinfield

It has been generally accepted that these three types of organism can be used for the measurement of vitamin B_{12} itself, and for some time now we have been using all three for work in our laboratory. We soon observed, however, that they respond differently to certain vitamin B_{12}-like factors, and we now use them in conjunction as a means of differentiation between these factors.

For assays with *Lb. leichmannii* our method and medium are essentially as described by Skeggs *et al.* (1950) but with certain minor modifications in technique. In our experience this method measures in a reproducible fashion those alkali-labile substances in cyanide extracts of a wide variety of natural materials active for *Lb. leichmannii*.

Our *Bact. coli* method is that of the Glaxo workers (Bessell *et al.* 1950; Cuthbertson *et al.* 1951; Harrison *et al.* 1951). With exceptions discussed below, we find broad similarity between results obtained with *Bact. coli* and *Lb. leichmannii*, and for certain crude and refined liver extracts agreement is good.

For the *Euglena gracilis* assays we use a technique based on that of Hutner *et al.* (1949). Results obtained for liver extracts agree broadly with those obtained with *Bact. coli* and *Lb. leichmannii*, but in general reproducibility is not satisfactory. In our hands the method has so far proved incapable of precisely measuring the vitamin B_{12} activities of liver preparations, fish and whale products and gut contents and faeces. We have used the method allied with chromatography to indicate qualitatively the presence of vitamin B_{12} activity in extracts.

Properties of some microbiologically active substances related to vitamin B_{12}

Natural materials may contain substances of the vitamin B_{12} group active for one or more assay organisms (Coates, Ford, Harrison, Kon & Porter, 1952; Ford *et al.* 1951; Ford & Porter, 1952; Kaczka *et al.* 1950; Smith *et al.* 1951; Pfiffner *et al.* 1951; Pierce *et al.* 1949; Tarr, 1951; Wijmenga, 1951). Further compounds closely related to vitamin B_{12} have been prepared in the laboratory (Cooley, Ellis, Petrow, Beaven, Holiday & Johnson, 1951; Kaczka *et al.* 1950; Smith *et al.* 1951). Though most of these substances can be converted to vitamin B_{12} by the action of cyanide, we have found in calf gut-contents and faeces, in addition to vitamin B_{12}, three substances, provisionally described as fractions A, B and C, related to vitamin B_{12} but having widely different activities for different assay organisms, and not convertible by cyanide (Coates, Ford, Harrison, Kon & Porter, 1952; Ford *et al.* 1951; Ford & Porter, 1952). Fractions A and B are red, cobalt-containing pigments. Fraction C is found in calf faeces but has not yet been isolated from this source. We have prepared it by growing *Bact. coli* in a vitamin B_{12}-free medium supplemented with fraction B. The vitamin B_{12} activity of extracts of the harvested cells was in the form of fraction C. Both fraction A and vitamin B_{12} were recovered largely unchanged after such passage through *Bact. coli*.

Fraction A is about as active for *Bact. coli* and *Euglena gracilis* as vitamin B_{12}. It is the most abundant active component in cyanide extracts of calf faeces and is approximately five times more active for *Bact. coli* and *Euglena gracilis* than for *Lb. leichmannii*. Modification of the *Lb. leichmannii* assay-medium by omission of thiomalic acid and inclusion of sodium cyanide (cf. Soars & Hendlin, 1951) results in an apparent marked increase in the activity of fraction A relative to vitamin B_{12}.

Fraction B is probably as active for *Bact. coli* as vitamin B_{12}, but it has less than $\frac{1}{2000}$ of this activity for both *Lb. leichmannii* and *Euglena gracilis*, and may be quite inactive for them. There is marked non-parallelism in the *Bact. coli* assay of this substance in terms of vitamin B_{12}.

As far as we can judge, fraction C is as active for *Bact. coli* as fraction B and in crude extracts has about one-fifteenth this activity for *Lb. leichmannii*.

Fractions B and C produce similar and characteristic zones of exhibition on *Bact. coli* plates. They are less sharply defined than those characteristic of fraction A and vitamin B_{12}.

We have prepared a derivative of fraction A apparently analogous to vitamin B_{12b} (Ford & Porter, 1952). It may well be that further compounds exist, related to fractions A, B or C in the same way as the cobalamin series is related to vitamin B_{12}.

Cyanide treatment of these three fractions had no effect on their chromatographic behaviour or on their various microbiological potencies.

Fractions A and B are inactive for the chick when given in the diet, but fraction A is active by injection, and on clinical test was shown to be about as active as vitamin B_{12} in a single case of pernicious anaemia (Coates, Ford, Harrison, Kon & Porter, 1952).

Pfiffner *et al.* (1951) have isolated a rumen organism that in anaerobic fermentation produced a mixture of cobalt-containing pigments of the vitamin B_{12} group. They crystallized two of these substances, which were active for *Lb. leichmannii* and *Lb. lactis* but inactive in chick and rat tests.

Wijmenga (1951) obtained a compound, vitamin B_{12m}, from the faeces of pigs, horses and cattle. Its absorption spectrum was similar to that of vitamin B_{12}, but it was slower-moving on paper chromatograms, and on microbiological assay with *Lb. leichmannii* had only about half the activity of vitamin B_{12}. Vitamin B_{12m} is a cyano-complex, and strong illumination of a solution in 0·001 N-H_2SO_4 gave rise to a substance of smaller R_F value. This derivative was designated vitamin B_{12mb}.

Another substance, 'factor WR', was isolated from liver. Its absorption spectrum coincided with that of vitamin B_{12}, but it behaved very differently in paper partition-chromatography, and had only 40% of the microbiological activity (*Lb. leichmannii*) of vitamin B_{12}.

Effect of cyanide in facilitating extraction of vitamin B_{12}-active substances from natural materials

De Heus & De Man (1951) have measured microbiologically the vitamin B_{12} potencies of a number of substances extracted in the presence and absence of cyanide. In several instances they obtained much higher results with cyanide. In our experience cyanide facilitates extraction of the vitamin B_{12}-active components of gut contents and faeces, and gives higher and more reproducible results than those obtained without cyanide. Cyanide treatment did not increase the potencies of extracts, whereas its addition before extraction gave extracts of greatly increased potencies (Coates, Ford *et al.* 1951).

Wijmenga *et al.* (1950) showed that cyanide can replace proteolytic enzymes in the extraction of vitamin B_{12} from liver, as it apparently removes a protein or peptide group, originally linked to the vitamin B_{12} molecule. We have compared different methods of extracting vitamin B_{12} from pig livers and found that steaming or autoclaving the homogenized livers with buffer at pH 5·5 in the presence of cyanide was as effective, as judged by *Lb. leichmannii* assay, as was autolysis followed by digestion at 65° with cyanide-activated papain. *Bact. coli* assays of the papain digests were unsatisfactory because of the large amounts of interfering substances present. No such interference was found with extracts prepared by the other methods, and with these extracts the two methods of assay gave similar results.

Assay of preparations containing mixtures of active substances that are not readily converted to vitamin B_{12} *on cyanide treatment*

To assay preparations containing mixtures of active substances, it may be necessary to fractionate and assay the substances separately against appropriate standards, and chromatographic methods have obvious applications to this end. We have described a technique for the rapid separation on paper chromatograms of the vitamin B_{12}-active substances in gut contents and faeces (Ford *et al.* 1951). We have used it to study the increase in the vitamin B_{12} potency of calf faeces following incubation, shown by chick tests. The microbiological potency for *Lb. leichmannii* changed little on incubation, whereas potency for *Bact. coli* diminished. We found that incubation resulted in a marked increase in the vitamin B_{12} component and the disappearance of a proportion of the activity contributed by other factors, in particular fraction A. This evidence suggests a possible explanation of the discrepancies between chick and microbiological assays, and of the increased potency for chicks after incubation.

'*Binding*' *of vitamin* B_{12}

Some natural materials have the property of apparently combining with vitamin B_{12} and making it unavailable to assay micro-organisms (Bird & Hoevet, 1951; Meyer *et al.* 1950; Shaw, 1950; Ternberg & Eakin, 1949). Sow's milk, for example, ties up considerable amounts of added vitamin B_{12}. The binding factor is not present in ultra-filtrates of sow's milk, and vitamin B_{12} added to the milk becomes not ultra-filtrable. The apparent inhibition of growth of the assay organisms is overcome by the addition of vitamin B_{12} in excess of about 0·05 μg/ml. milk. The binding property is not destroyed, nor is added vitamin B_{12} released, on autoclaving the milk for 15 min at 120°. Digestion with cyanide-activated papain, or autoclaving in the presence of a trace of cyanide, however, destroyed the 'binding' property of the milk and released added vitamin B_{12} (Gregory, Ford & Kon, 1952).

Conclusions

The non-specificity of available techniques of microbiological assay can complicate their use with some natural materials that may contain substances related to vitamin B_{12}, but differently active for different micro-organisms. It is possible, however, that these substances are less readily utilized by higher animals than is vitamin B_{12} itself and that the assay of animal tissues may therefore present fewer problems of non-specificity than that of fermentation products.

The use of *Bact. coli* in bioautographic techniques offers a ready means of identifying at least three substances related to vitamin B_{12} that may be encountered in cyanide-treated extracts of natural materials. The presence of cyanide can facilitate the extraction of vitamin B_{12}-active substances from natural materials and simplify their measurement.

When microbiological assays are used to assess the value of foods for man or beast, it should be borne in mind that the response of micro-organisms to any nutrient as present in natural materials is not necessarily that of higher animals and, as with all assays, the question turns on the purpose for which the assay is carried out (Coates, Ford, Harrison, Kon, Shepheard & Wilby, 1952).

Reports of marked discrepancies between results of biological and microbiological assays of vitamin B_{12} and related substances (Coates, Harrison & Kon, 1951; Combs, Carlson, Miller, Peeler, Norris & Heuser, 1950; Lewis, Tappan & Elvehjem, 1950; Pfiffner *et al.* 1951) further emphasize the need for a biological check before interpreting the results of microbiological assays in terms of animal requirements.

With microbiological assays of vitamin B_{12} it appears that the fullest particulars of methods of extraction and assays, with data on recovery tests and possibly also the protocols of a typical assay, may be necessary for the interpretation of published findings. To quote Harris (1949), 'It is essential that workers reporting the results of vitamin determinations should not be content with giving merely a numerical value, but should state also the conditions employed for the assay'.

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The Nutritional Significance of the Animal Protein Factor and Antibiotics

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Diets composed wholly of vegetable materials have for some time been known to be inadequate for the growth of chicks (Hammond & Titus, 1944*a*, *b*; Rubin & Bird, 1946) or rats (Zucker & Zucker, 1948), even when supplemented with all the previously recognized vitamins. The factor (or group of factors) essential for the growth of animals maintained on these diets came to be called the 'animal protein factor' (APF) and was soon shown, though not a protein, to be present in materials of animal origin, e.g. milk, meat, liver, fish meal and cow manure (Rubin, Groschke & Bird, 1947; Bird, Rubin & Groschke, 1948).

The growth of chicks and pigs on wholly vegetable diets was greatly improved by administration of vitamin B_{12} (Ott, Rickes & Wood, 1948; Hogan & Anderson, 1949), and in experiments with vitamin B_{12} it was found to be equivalent to a liver fraction or to 'fish solubles'. Several other workers (Sunde, Cravens, Elvejhem & Halpin, 1950; Hill & Branion, 1950; Swenson, 1951) reported that vitamin B_{12}, even when given with all other known growth factors, could not fully replace the effects of foods of animal origin in the diets of chickens and turkeys. These discordant findings can now be explained by considering the previous history of the experimental animals, for Coates, Harrison & Kon (1950) demonstrated that vitamin B_{12} could fully replace animal protein if chicks from normal hens or hens recently placed on a deficient diet were used, whereas for chicks derived from hens maintained for some months on the deficient diet vitamin B_{12} alone was not fully effective.

Stokstad, Jukes, Pierce, Page & Franklin (1949) showed that supplementation of an all-vegetable ration with certain fermentation products, used as a source of vitamin B_{12}, led to much better growth than could be obtained even with superoptimal amounts of vitamin B_{12}; indeed, the growth was better than with vitamin B_{12} and a source of animal protein factor, such as fish solubles, given together. Further work showed that the growth-promoting activity of this fermentation product was associated with its aureomycin content, and that supplementation with both aureomycin and vitamin B_{12} was as effective as the whole fermentation product in promoting growth of chicks on rations deficient in animal protein (Stokstad & Jukes, 1950). Other antibiotics also, in particular penicillin, terramycin and bacitracin, were soon shown to be effective in promoting the growth of chicks, rats, turkeys and pigs maintained on wholly vegetable diets supplemented with vitamin B_{12} (Cuthbertson, 1952).

That the antibiotics exert their effects through an action on the gut flora seems probable for the following reasons:

(1) The active concentrations in the food are so low that antibacterial concentrations in the tissues are unlikely to result.

(2) Injected antibiotics are not as effective as those orally administered.

(3) Antibiotic activity appears to be the only property shared by this chemically diverse group of substances.

Aureomycin, terramycin, penicillin and streptomycin were found to increase the growth of chicks even on excellent rations containing ample animal protein (Coates, Harrison, Kon, Mann & Rose, 1951; Biely, March, Stevens & Casorso, 1951). Aureomycin has also been shown to improve the growth and survival of premature infants (Robinson, 1952).

Foods of animal origin (APF) improve growth on vegetable rations because they contain vitamin B_{12} and other undescribed growth factors. Some antibiotics, when given with vitamin B_{12}, also increase growth and in most instances supplements of this type produce even better growth than do unsupplemented rations containing APF in food of animal origin. It is not known whether antibiotic supplements can fully replace exogeneous APF over the whole life cycle.

The use of the term APF is perhaps best avoided when referring to supplements that depend, for some or all of their effects, on their antibiotic content. The description APF should thus be reserved for those supplements, free from antibiotics, such as foods of animal origin that promote growth on wholly vegetable rations. This should prevent any confusion that could arise between the two types of supplement, which do differ in their modes of action and certain of their effects. The investigation of the unidentified factors in foods of animal origin is complicated because of the possible effects of the gut flora on their synthesis (or destruction) and the probability that the factors can be transferred through the egg, or the placenta and milk, to the next generation. The use of germ-free animals would appear to offer great advantages in investigation of these factors, but even with their use the problem of characterizing the factors would be difficult.

In our laboratories penicillin was found to have no effect on the growth of chicks offered rations containing animal protein, whereas penicillin always increased the growth rate of chicks in the laboratories at the National Institute for Research in Dairying, at Shinfield. To investigate this discrepancy a joint experiment was carried out. The same batch of food and chicks from the same hatch were shared between the two laboratories. Similar results were again obtained; in that at Shinfield the birds receiving penicillin grew better than the others, whereas at Greenford the birds grew equally well, with or without the addition of penicillin to their food, as can be seen in Table 1. The observation that the growth of the birds at Greenford on either regime was as good as or better than the growth of the penicillin-treated birds at Shinfield led to the hypothesis that the latter suffered from some condition depressing growth and eliminated by dietary penicillin. Further work (Coates, Dickinson, Harrison, Kon, Porter, Cummins & Cuthbertson, 1952) has clearly demonstrated the presence in the Shinfield laboratories of a highly infectious condition, overcome by oral penicillin and best described as 'the inability to grow adequately in the absence of dietary antibiotics'. Direct contact between birds is not necessary for spreading of this infection, which is not transmitted through the egg.

Table 1. *The effect of penicillin on the growth of chicks on National Baby Chick Mash**

(Mean weights (in g) at 3 weeks of age of groups of twenty Rhode Island Red × Light Sussex cockerels)

	Procaine penicillin (mg/kg diet)	
Laboratory	0	40
National Institute for Research in Dairying, Shinfield	167	192
Glaxo Laboratories, Greenford	188	191

* Coates, Dickinson, Harrison, Kon, Cummins & Cuthbertson (1951).

The condition is not associated with any increased mortality (the death rate in both laboratories is about the same and less than 3 %) or with any known poultry disease. The absence of this infection from Glaxo Laboratories may be explained by the previous complete exclusion of all adult poultry from those laboratories, for till recently birds were seldom kept, whereas at Shinfield birds have been continuously maintained for the past 10 years. The possibility that an infection of this type may be widespread is indicated by the many reports that antibiotics improve the growth of birds on normal diets: further support is given by Davis & Briggs (1950), who noted that in their new poultry houses growth of untreated birds appeared at first to be about the same as that of birds given antibiotics in their old poultry houses.

Although an infective condition of this type may explain many of the growth responses obtained with dietary antibiotics, it cannot explain all; thus, in our laboratory dietary penicillin has little effect on rats given milk-yeast-cereal diets, but invariably improves the growth of animals on vitamin B_{12}-deficient rations (Cuthbertson & Thornton, unpublished). Further, in our laboratory dietary penicillin increases the growth of chicks offered wholly vegetable rations even when the infective condition can be proved to be absent (Table 2 above; Cummins, Cuthbertson & Flynn, unpublished).

Table 2. *Effect of dietary antibiotics and vitamin* B_{12} *on growth of chicks maintained on vegetable rations in the absence of an infective condition depressing growth*

(Mean weights (in g), with their standard errors, at 5 weeks of age of groups of twelve Light Sussex × Rhode Island Red pullets)

Diet	Vitamin B_{12} (µg/kg diet)	Procaine penicillin (mg/kg diet)	
		0	40
All-vegetable	0	369 ± 13	401 ± 14
	200	422 ± 14	422 ± 15
National Baby Chick Mash	0	381 ± 16	381 ± 12

The different effects of dietary antibiotics on the growth of animals appear to depend to some extent on what has been called the 'disease level' (a better term would probably be 'microbiological status') of the animals studied.

(1) Antibiotics may often be effective by elimination of frank intestinal infections; thus, in much published work on pigs it is clear that the animals studied were suffering from scours, and in many instances the reported reduction of mortality and increased growth of chicks and turkeys may also have been due to elimination of disease. Speer, Vohs, Catron, Maddock & Culbertson (1950), for example, found antibiotics ineffective when given to pigs kept under first-class hygienic conditions.

(2) The elimination of growth-depressant infective conditions that depress growth but do not cause disease or increased mortality may also frequently be responsible for the beneficial effects of antibiotics (Coates, Dickinson, Harrison, Kon, Cummins & Cuthbertson, 1951; Coates *et al.* 1952).

(3) In the absence of conditions (1) and (2) antibiotics appear to have no effect on the growth of rats and chicks receiving adequate diets, though improvements are still obtained with animals fed on all-vegetable rations (Cummins, Cuthbertson, Flynn & Thornton, unpublished).

(4) The effect of antibiotics in the complete absence of intestinal micro-organisms is not yet known and its study should be of great interest.

The amounts of antibiotic required are very small—2–15 g/ton of food eaten—and only small changes have been noted in the gut flora on applying the classical methods of bacteriology.

The presence of antibiotics could modify growth in several different ways, thus:

(i) by elimination of known pathogens;
(ii) by elimination of micro-organisms depressing growth,
    (*a*) that produce toxins,
    (*b*) that compete for essential nutrients;
(iii) by modification of bacterial metabolism or production of antibiotic-resistant strains with modified metabolism,
    (*a*) with reduced requirements for essential nutrients,
    (*b*) without ability to adsorb essential nutrients,
    (*c*) with increased powers of synthesis or secretion of essential nutrients;
(iv) by elimination of antibiotic-sensitive organisms, leading to increased growth of other types, which could either,
    (*a*) not compete for essential nutrients,
    (*b*) synthesize growth factors.

Apart from their effects on frank disease and the infection that depresses growth, antibiotics modify growth by improving the efficiency of utilization of certain vitamins in both the chick and the rat (Coates, Dickinson, Harrison & Kon, 1951; Lih & Baumann, 1951; Biely & March, 1951). This vitamin-sparing action, at least for vitamin B_{12}, is demonstrable even in the absence of any growth-depressant infection (Cummins, Cuthbertson & Flynn, unpublished).

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The Animal Protein Factor in the Nutrition of Chickens

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The use of the chicken for the study of the animal protein factor (APF) has brought to light several properties of vitamin B_{12} and related substances, and has contributed largely to the recognition of their significance in animal nutrition, apart from their function as anti-pernicious-anaemia factor(s). In particular, their importance in reproduction and growth has been studied with poultry.

Properties of the animal protein factor

Effect in reproduction. Many workers in the past have postulated the existence of a 'hatchability factor' essential for the successful hatching of fertile eggs from hens on all-vegetable diets. Among others, Bird, Rubin, Whitson & Haynes (1946) reported improvement in hatchability with supplements of fish meals, dried milk, fermentation products and dried cow manure; Lindstrom, Moore, Petersen, & Wiese (1949) obtained similar improvements with fish meal and a concentrate from cow manure, and Black, Getty, Coates, Harrison & Kon (1950) with a vitamin B_{12} concentrate (Examen, Glaxo Laboratories Ltd.). That the active material was in fact vitamin B_{12} was shown by Lillie, Olsen & Bird (1949), who injected solutions of the crystalline vitamin into eggs from hens on vegetable diets during incubation and obtained a marked increase in the number hatched. Carver & McGinnis (1950) confirmed their observation by injecting 2 μg vitamin B_{12} into deficient eggs before incubation with a consequent increase in hatchability from 35 to 90 %. Olcese, Couch, Quisenberry & Pearson (1950) have described signs of vitamin B_{12} deficiency in the developing embryo, including atrophy of the leg muscles, multiple haemorrhages and perosis. These authors also confirmed our own experience that embryos die most frequently about the 17th day of incubation.

As yet there is no evidence that APF deficiency has adverse effects on fertility of either male or female.

Effect on growth. It is now generally accepted that a source of the APF is essential in the diet of the growing chick. The literature on the subject is too extensive to be quoted in detail; early work has been reviewed by Schweigert (1949). It is our own experience, and that of many other workers, that hens given normal diets pass on to their progeny sufficient reserves of the APF to last through the initial growing stages. It is probably this transference of reserves from mother to offspring that has delayed for so long the discovery of the animal protein factor; its existence was only recognized when plant sources of proteins came into extensive use in American poultry rations, resulting in poorer hatchability and viability of the young chicks.

The chick, being a rapidly growing animal, very soon shows signs of a lack of essential growth factors and although, in contrast to man, no anaemia is evidenced, a severe deficiency of the APF results in very poor growth, poor feathering and usually death in 3–4 weeks. The chick appears unable to compensate to any extent for the deficiency by the activity of its intestinal micro-organisms. These facts make the chick a very suitable subject, not only for the biological assay of the APF, but also for the fundamental study of its various components.

The chick assay of the animal protein factor

A number of workers have used chicks for the biological assay of the APF (Lillie, Denton & Bird, 1949; Nichol, Robblee, Cravens & Elvehjem, 1947; Stokstad, Jukes, Pierce, Page & Franklin, 1949; Coates, Harrison & Kon, 1950, 1951), all based on the use of chicks from hens themselves depleted of the APF. We have found it helpful to include a small amount of vitamin B_{12} as Examen (Glaxo Laboratories Ltd.), equivalent to about 0·25 μg vitamin B_{12}/100 g, in the diet of the hens in order to maintain the hatchability of their eggs at a reasonable level, while only negligible reserves are passed on to the chicks hatched from them. Our method consists essentially of maintaining groups of about ten depleted chicks on three to four dose levels of test material (incorporated into the basal vegetable-protein diet) and comparing their weight responses with those of similar groups given a standard preparation. Robblee, Nichol, Cravens, Elvehjem & Halpin (1948) recommend the inclusion of thyroprotein in the chicks' diet, thereby increasing their requirement for the APF and extending the effective range of the dose-response curve. We have examined the accuracy of our method and found that variance between birds on the same doses was very high, possibly as a result of difference in depletion rates of their dams. The variance can, to some extent, be overcome (Lillie, Denton & Bird, 1949), by keeping the chicks for up to 14 days on the basal diet and rejecting those falling outside defined weight limits at the end of the depletion period.

In spite of the high inherent error, we have found the results of chick assays surprisingly reproducible (Coates *et al.* 1950). However, they rarely agree with those found by microbiological techniques. Broadly speaking, chick assays give much higher results for animal-protein products such as fish and whale solubles, and markedly lower values for rumen and faecal materials (Coates, Ford, Harrison, Kon, Porter, Cuthbertson & Pegler, 1951) and these discrepancies add to the already considerable evidence accumulating to show that the APF is not indeed a single entity but a multiplicity of factors with different activities for different organisms.

Evidence, with chicks, for the complex nature of the APF

When vitamin B_{12} was first obtained in pure form it was considered by some (Ott, Rickes & Wood, 1948; Nichol, Dietrich, Cravens & Elvehjem, 1949; Lillie *et al.* 1948) to be itself the APF, as its addition to the diet of chicks on all-vegetable rations resulted in considerable gains in weight. Its maximal effect was produced with doses of 3 μg/100 g diet (Ott *et al.* 1948). More recently, however, many reports indicated that chick growth can be further improved in the presence of optimal amounts of vitamin B_{12} by supplements of crude materials such as fish solubles or certain samples of dried brewer's yeast (Carlson, Miller, Peeler, Norris & Heuser, 1949; Arscott & Combs, 1950), distiller's dried solubles (Novak, Hauge & Carrick, 1947) and crude liver extracts or dried whey (Menge, Combs & Schorb, 1949).

Although it is possible that the discrepancies reported above between chick and microbiological tests of such materials are due to incomplete extraction in the microbiological assay, it is more likely that the high values found by the chick method reflect the presence in the test materials of unidentified nutrients essential for, or utilized by, the chick but not the micro-organisms employed in the assay.

In the course of our chick assays we have obtained two further indications of the multiple nature of the APF (Coates *et al.* 1950). Firstly, we have had little success in obtaining linear dose-response curves to crystalline vitamin B_{12}. A highly refined liver extract such as Examen (Glaxo Laboratories Ltd.) gives more satisfactory responses, but much better linearity is obtained with crude material such as fish solubles. It is, therefore, probable that the crude materials contain a factor necessary for the proper utilization of vitamin B_{12} by higher animals. This possibility has been strengthened with the isolation by Ford & Porter (1952) of a vitamin B_{12}-like substance from calf faeces that, although itself devoid of APF activity for chicks, considerably enhances their response to pure vitamin B_{12} (Coates, Ford, Harrison, Kon & Porter, 1952). We have also found that certain preparations from preserved herrings possess the same property. The substance is not identical with the 'intrinsic factor' of human pernicious anaemia because we have found a sample of freeze-dried normal human gastric juice ineffective in enhancing the chick's response to added vitamin B_{12}.

Secondly, we have found that the APF activity of whale and fish solubles, as measured by chicks, increased with the degree of depletion of their dams. Assays of a sample of whale solubles done simultaneously with chicks from hens freshly depleted and those from hens that had received a vegetable diet for at least a year gave values of 0·14 and 0·70 μg vitamin B_{12} activity/g respectively (Coates *et al.* 1950). Similarly, the apparent vitamin B_{12} activity of a sample of fish solubles was 1·3 μg/g with one hatch of chicks and well over 3·0 μg/g with a hatch from the same hens 6 months later. From this evidence we believe that hens given all-vegetable diets become depleted first of vitamin B_{12} and later of another member (or members) of the APF complex, and that these successive depletions are reflected in the response of the offspring to sources of the APF.

The spate of reports that so-called 'APF concentrates' prepared as by-products in the manufacture of antibiotics have growth-promoting activity for chicks beyond that of their vitamin B_{12} content culminated in the finding (Stokstad & Jukes, 1950) that this activity was due to the presence of traces of antibiotic remaining in the concentrates. Coates, Dickinson, Harrison, Kon, Porter, Cummins & Cuthbertson (1952) discuss the question whether the growth stimulation is a direct effect of the antibiotic itself or occurs indirectly as the result of increased synthesis of components of the APF in the gut of the chick, but the position is too confused at present to contribute much towards elucidating the identity of the APF.

In spite of the considerable weight of evidence for the existence of unidentified essential nutrients in the APF complex, there are still some who maintain that maximal growth in chicks can be obtained when crystalline vitamin B_{12} is the sole APF supplement. Thus Ott (1951), using a vegetable diet containing 2 μg vitamin B_{12}/100 g, was unable to improve chick growth by addition of whey, fish solubles or a crude liver fraction. The basal diet used in Ott's experiments contained very large supplements of glycine, arginine, methionine, cystine and choline and if, as seems probable, the APF is concerned in the metabolism of protein, it is likely that large supplements of pure amino-acids might reduce the animal's need for at least some of the APF components.

The biochemical significance of the APF in chick nutrition

The biochemical functions of the APF are still obscure. That it is involved in the metabolism of proteins is apparent, as an increase in the protein content of chick diets lacking APF accentuates the signs of its deficiency in the chicks (Rubin & Bird, 1947). McGinnis, Hsu & Graham (1948) found higher than normal levels of non-protein-nitrogen in the blood of chicks deprived of APF and suggested that the APF was concerned in the utilization of amino-acids. Their suggestion received further support from Charkey, Wilgus, Patton & Gassner (1950), who measured microbiologically arginine, lysine, methionine, tryptophan, histidine, threonine and valine in chick blood and found higher values for all seven amino-acids in the blood from depleted chicks as compared with controls given vitamin B_{12}.

There are numerous indications from work with chicks that the APF is concerned in transmethylation processes. The suggestion was first made by Gillis & Norris (1949*a*) who showed that in supplying the chick's need of APF in the form of a liver paste they reduced its requirement for choline and betaine. Later the same authors (Gillis & Norris, 1949*b*) found that crystalline vitamin B_{12} had the same sparing action on methylating compounds. Similarly, Schaefer, Salmon & Strength (1949) demonstrated that the choline requirement of APF-deficient chicks was reduced six-fold when vitamin B_{12} was added to their diet. Later, the same authors (Schaefer, Salmon, & Strength, 1951) showed that certain precursors of choline, in particular dimethylaminoethanol, could be utilized by chicks in place of choline itself, but only if vitamin B_{12} was present in the diet. Briggs, Hill & Giles (1950) also studied the relationship between choline and vitamin B_{12} in chicks, and although they found that choline exerted a sparing action on the birds' requirement for vitamin B_{12}, DL-methionine was far more efficient in this respect and they concluded that, under certain conditions at least, chicks may do without extraneous vitamin B_{12} if sufficient methionine is present in the diet. Patrick (1950) arrived at similar conclusions, but Jukes & Stokstad (1951), though they confirmed the sparing action on vitamin B_{12} of both methionine and choline, were unable to produce optimal growth in chicks without both methionine and vitamin B_{12}. The differences in results obtained by different workers can undoubtedly be explained by slight variations in experimental conditions.

The relationship between vitamin B_{12} and other vitamins has been investigated in experiments with chicks by a number of workers. Schaefer, Salmon, Strength & Copeland (1950) postulated the interrelationship of folic acid, vitamin B_{12} and choline. Dietrich, Monson & Elvehjem (1951) presented data demonstrating that vitamin B_{12} is concerned in the conversion of folic to folinic acid in the chick, but we ourselves have been unable to diminish signs of vitamin B_{12} deficiency in chicks by injections of folinic acid. Others (Yacowitz, Norris & Heuser, 1951) have reported a mutual sparing action between pantothenic acid and vitamin B_{12}. The interrelationship of other vitamins or amino-acids and vitamin B_{12} may account for some of the evidence for the complexity of the APF. No doubt, with the identification of the components of the APF its biochemical significance will be explained, and it is clear that work with chicks can throw considerable light on the problem of the nature and functions of the APF.

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Nicotinic Acid, Pantothenic Acid and Biotin in Fruits, Vegetables and Nuts

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(*Received* 8 *November* 1950)

Little information is available about the vitamins of the B group (with the possible exceptions of aneurin and riboflavin) in fruits and vegetables, particularly those most widely cultivated in the British Isles. The riboflavin contents of home-grown fruits have been surveyed by the author (James, 1944). This paper records estimations, made in the 1947 and 1948 seasons, of nicotinic acid, pantothenic acid and biotin in fruits, vegetables and nuts commonly found in English gardens and hedgerows.

EXPERIMENTAL

Materials and methods

During the 1947 season assays were made on fourteen kinds of fruit, comprising thirty varieties. With the exception of cherries, all fruits were grown in the Research Station plantations under normally good conditions of cultivation. Soft fruits, stone fruits and gooseberries were picked at the best stage of maturity for cooking or dessert, depending on variety, and extraction for assay was begun within the hour. Pears were picked when slightly immature and ripened at room temperature. Apples of keeping varieties were sampled 1 week after picking in October and again after storing at 36° F for 10–12 weeks, when they were fully ripe. The cherries came from Kent and were assayed not more than 24 h after picking.

In 1948 some twenty-three different vegetables and seven kinds of nuts were assayed at the stage of maturity considered to be best for palatability. Most of the vegetables were grown at the Research Station and used immediately after gathering. A few were obtained locally and the asparagus was grown in the Evesham district, the assays being made 24 h after harvesting.

The nuts were obtained from hedges, gardens and plantations in Somerset and Kent, and were allowed to ripen in their shells at room temperature before assay.

Measurements

Nicotinic acid, pantothenic acid and biotin were assayed by microbiological methods based on those published by Barton-Wright (1944, 1945, not dated); the test organism was a subculture of the strain of *Lactobacillus arabinosus* used by him. The development of microbiological assay methods has made it possible to undertake certain vitamin estimations in laboratories where such work was hitherto not attempted because of the lack of suitable chemical methods or of the time, space and labour required for biological assays. The varying degree of success with which microbiological methods have met appears to have been due to variations in technique, equipment, reagents and treatment of the test organism; these have not always been reported or even appreciated by the worker.

There also remains the fact that we know the microbiological methods to work, but that the precise nature of cell activity involved in an assay is still uncertain. A detailed account of the methods used is therefore given here, so that the accuracy of the results obtained can be assessed and compared with those of other workers at the present time and in the light of future knowledge.

Sampling

Fruits. A representative sample (at least six apples or pears), sufficient to give about 500 g prepared edible portion, was taken from each batch of fruit.

Soft fruits were hulled and washed when necessary; stone fruits were wiped with a dry cloth, stems and stones being then removed; hard fruits were peeled and cored; gooseberries were topped and tailed; hairs and seeds were removed from rose-hips, and tomatoes were merely wiped.

Vegetables. As with the fruits, each lot of vegetables was sampled so as to give about 500 g edible portion for extraction. Peas and dwarf beans were shelled, the seeds only being used, and runner beans were wiped and both pods and seeds used. Leafy vegetables were washed and wiped and inedible parts of the stalk removed. For large leafy vegetables, such as savoy cabbage and cabbage lettuce, longitudinal quarters or halves from several plants were bulked to form the sample. Cauliflowers were similarly sampled and divided into flower, leaf (including edible stalk and midrib) and inedible stalk. Roots and tubers were washed and scraped or peeled, according to custom; when individual vegetables were large, longitudinal quarters from at least six specimens were again taken. Celery heads were halved longitudinally and several halves were divided into stem and leaf samples, which were washed and wiped before using. Samples of asparagus included tips and edible stems, whereas only the flesh of marrow was used, the method of longitudinal sectioning being again employed. About 2 in. of edible green leaf were included in samples of leeks; the outer brown layers of onions were removed.

Each bulk sample of fruit or vegetable was either minced in a stainless domestic-type machine or macerated in a Waring Blendor. At least two 10–50 g portions of the thoroughly mixed sample were weighed for assay.

Nuts. The kernels were peeled and about 5 g finely chopped material were ground in a mortar with fine, purified sand.

Extraction

Observations by the author (James, 1947) and others, confirmed by Kodicek & Pepper (1948), have shown *Lb. arabinosus* to be relatively insensitive to stimulation by fats and fatty acids when compared with *Lb. helveticus*, which is used in the assay of riboflavin. With materials of high fat content such as nuts it was, however, found necessary to extract samples for 10 h with ether in a Soxhlet apparatus before attempting aqueous extraction of the vitamin. From the defatted bulk sample of each variety samples were taken for vitamin extraction.

The following simple procedure was adopted; by its means assay of the three vitamins could be made on a single extract. The same extract could also be used for the assay of riboflavin with *Lb. helveticus*.

Two weighed portions of the minced bulk sample were finely blended with 40 ml. acetate buffer (pH 4·5), transferred into 600 ml. conical flasks, covered with inverted beakers and autoclaved for 15 min at 15 lb. pressure. After cooking, 20 mg of pure papain (Ferris & Co) and 20 mg takadiastase (Parke, Davis & Co.) were added to each fruit extract and ten times that quantity to nut and vegetable extracts. When several samples were extracted the total amount of enzymes required was blended with a few drops of glycerol and made up to a convenient volume with distilled water. Appropriate volumes of this enzyme preparation were then pipetted into each extract. Vegetables that had proved difficult to macerate before autoclaving, and those that had formed a thick sludge or starchy gel during autoclaving, were further ground before being blended with the enzymes in a mortar. Each extract was covered with a layer of toluene and incubated at 37° overnight (16–18 h). At first blank extracts, containing only buffer and enzyme preparation, were prepared and assayed in the same way as test extracts. As they consistently gave negligible values for nicotinic acid, biotin and pantothenic acid, this procedure was discontinued as a routine measure, but repeated occasionally. When extracts were to be assayed also for riboflavin, enzyme blanks were required.

After incubation the extracts were steamed for 30 min to stop enzyme activity. The deposit was removed by squeezing the hot incubate through double muslin; it was then washed well with distilled water, the whole aqueous extract being collected in a graduated flask. After cooling the pH was adjusted to 6·8 (bromothymol blue) with sodium hydroxide and acetic acid, and the extract was made up to the required volume. At this stage all extracts were shaken with chloroform, the chloroform layer being then centrifuged off and the clear aqueous layer retained for assay. Shaking with chloroform may or may not have been necessary for removal of fat and fatty acids, but it provided a safe and simple means of clarifying the extracts. Extracts so prepared were clear and generally colourless. In others the very light colour remaining did not interfere with subsequent titration of lactic acid. Usually extracts were assayed immediately after preparation, but it was found that they could be kept at 4° under toluene in close-stoppered bottles for 3 weeks without loss of vitamin.

Microbiological assay

Each extract was assayed at three or more different levels and triplicate readings were made at each level. For each vitamin, standard curves were obtained from a series of twenty-one flasks containing the vitamin at seven different concentrations.

It was found convenient to use 50 ml. conical flasks for all assays. These had the advantage over test-tubes that the final titration could be made directly in them; they were also easier to fill. Into each assay and standard flask exactly 5 ml. of the appropriate double-strength basal medium were run from a burette, a suitable volume of the extract under test, or of the pure vitamin in solution, was added by pipette, and the volume in each flask was brought up to 9·5 ml. with distilled water.

No difference was found in assays when the normal laboratory supply of water from tin-lined copper stills was used instead of the glass- or porcelain-distilled water recommended by other workers. Analyses of this water showed an average per litre of 0·004 mg iron, 0·004 mg manganese, 0·014 mg copper, 0·0075 mg zinc, 0·003 mg boron and 0·00035 mg molybdenum. The quantity of copper was evidently too low to interfere with the growth of *Lb. arabinosus*, and the ordinary laboratory supply of distilled water was used in the preparation of all extracts and mediums. The same result had previously been found in *Lb. helveticus* assays of riboflavin.

The flasks were plugged with cotton-wool of different colours to distinguish assays of different vitamins, with various combinations of colours to indicate various levels of concentration. All standard and assay flasks for the same vitamin were autoclaved together for 15 min at 15 lb. pressure. With this treatment the biotin medium remained almost colourless and the nicotinic-acid and pantothenic-acid mediums pale yellow. Longer sterilizing even at a lower temperature caused the mediums to darken, making subsequent titration difficult.

The basal medium used in assay of each vitamin was that recommended by Barton-Wright (not dated), the pH being always adjusted to 6·8. Details are given in Table 1. Both commercially prepared vitamin-free spray-dried casein hydrolysate and a casein hydrolysate prepared here have been used with equal success. When in 1947 this work was due to begin, no *p*-aminobenzoic acid was available and assay trials were made without it. The organism responded excellently and when later *p*-aminobenzoic acid was available its addition to the mediums produced no change in biotin and nicotinic-acid assays, but increased the production of acid in the assay of pantothenic acid, as shown by the following titrations:

	Growth response expressed in terms of approx. 0·1 N-NaOH	
Pantothenic acid (μg)	Medium without *p*-aminobenzoic acid (ml.)	Medium with *p*-aminobenzoic acid (ml.)
0·0	0·7	0·72
0·005	1·02	1·47
0·01	1·61	2·49
0·02	2·4	4·2
0·04	3·34	6·05
0·06	3·95	7·24
0·08	4·35	8·25

The strain of *Lb. arabinosus* employed would therefore appear to be the *p*-aminobenzoic acid mutant described by American workers and later by Kodicek & Pepper (1948). The inclusion of *p*-aminobenzoic acid in the mediums was continued in anticipation of the organism's reversion to type, but recent tests have shown that it is still able to dispense with *p*-aminobenzoic acid.

The inoculums were prepared by growing the organism overnight in basal mediums enriched with the missing vitamins. Stock cultures of the organism were carried on slopes of yeast-extract agar containing 1% glucose and were usually subcultured at weekly intervals. It was found unnecessary to hold them at 4° after incubation; cultures kept at room temperature for 3 weeks showed no change in response during assays. Two tapered centrifuge tubes were charged with 3 ml. nicotinic-acid assay basal medium, 3 ml. distilled water and 1·2 μg nicotinic acid. They were plugged and sterilized, and each was inoculated with a loopful of a 48 h slope culture of *Lb. arabinosus*. The two tubes were incubated at 37° for 16–18 h and the growths were then centrifuged aseptically, the supernatant medium being decanted off and the deposits in each taken up in about 6 ml. of sterile saline (0·9% NaCl solution). Aseptic spinning and washing in saline were repeated three times with each tube so as to remove all traces of medium. The final deposit in one tube was suspended in 6 ml. sterile saline and 2 ml. of this thick suspension were transferred to 50 ml. sterile saline to form the inoculum in the pantothenic-acid assay; the remaining 4 ml. were taken up in 50 ml. saline for assay of nicotinic acid. The deposit in the second tube was suspended in 50 ml. saline for biotin assay. This routine method of preparing the inoculums gave

Table 1. *Composition of basal mediums used in the microbiological assays. Quantities for fifty flasks (double strength)*

Nutrient	Strength of stock solution (wt./ml.)	For nicotinic-acid stock solution (ml.)	For biotin stock solution (ml.)	For pantothenic-acid stock solution (ml.)
Solutions:				
Photolysed peptone (Difco)	50 mg	—	—	50·0
L-Cystine	4 mg	25·0	12·5	25·0
DL-Tryptophan	2 mg	25·0	25·0	—
Adenine and guanine	1 mg	5·0	5·0	5·0
Adenine and uracil	1 mg	5·0	5·0	5·0
Xanthine	1 mg	5·0	5·0	5·0
Calcium D-pantothenate	1000 μg	0·05	0·1	—
Nicotinic acid	1000 μg	—	0·1	0·1
Riboflavin	50 μg	2·0	2·0	2·0
DL-Biotin	0·1 μg	2·0	—	2·0
Pyridoxine hydrochloride	100 μg	0·5	1·0	1·0
Aneurin	100 μg	0·5	0·5	0·5
p-Aminobenzoic acid	100 μg	1·0	1·0	1·0
Inorganic salt solution A:				
K_2HPO_4 (anhydrous)	100 mg	2·5	2·5	2·5
KH_2HPO_4 (anhydrous)	100 mg			
Inorganic salt solution B:				
$MgSO_4.7H_2O$	40 mg	2·5	2·5	2·5
$MnSO_4.4H_2O$	2 mg			
$FeCl_3$ (anhydrous)	0·4 mg			
Solids:				
Casein hydrolysate	—	3·0 g	1·5 g	1·0 g
Glucose (anhydrous)	—	10·0 g	5·0 g	10·0 g
Xylose (hydrated)	—	0·5 g	—	0·5 g
Sodium acetate (hydrated)	—	16·5 g	5·0 g	11·0 g
Sodium chloride	—	2·5 g	2·5 g	2·5 g
Ammonium sulphate	—	1·5 g	1·5 g	1·5 g
Water to	—	200 ml.	200 ml.	200 ml.
Adjust pH to 6·8 (bromothymol blue)				
Water to	—	250 ml.	250 ml.	250 ml.

surprisingly consistent and even suspensions, and with a little experience their opacity could be judged by the eye. A few check counts on a haemocytometer showed that best results were given by suspensions containing 12–15 million organisms/ml. for nicotinic-acid assay, 15–20 million/ml. for biotin assay, and 4–6 million/ml. for pantothenic-acid assay.

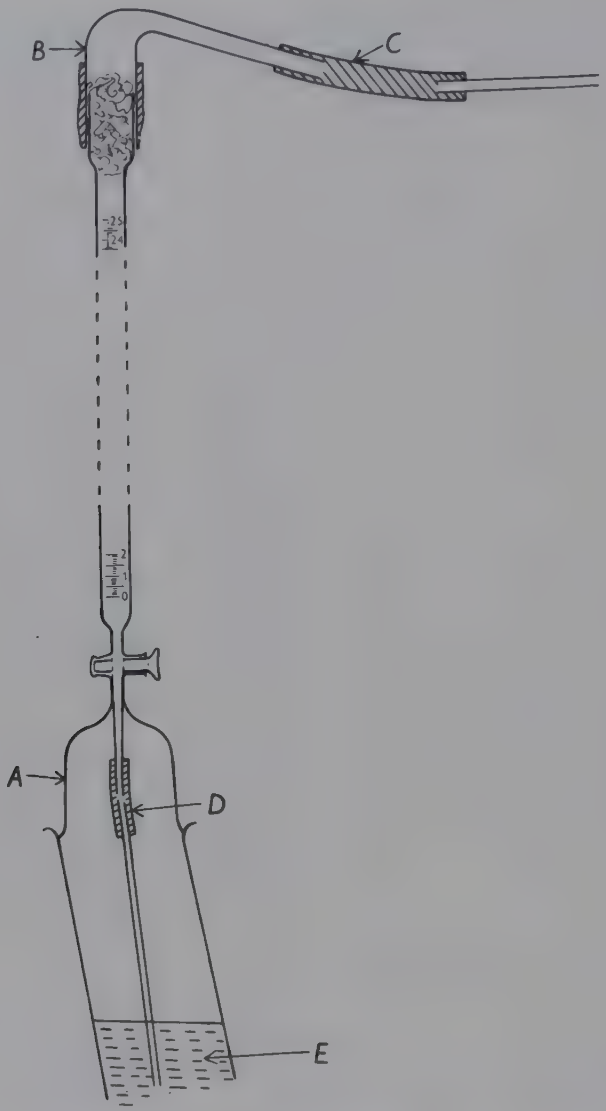

Fig. 1. Inoculating burette. For description see this page below.

Inoculation of each assay and standard flask was made with 0·5 ml. of the appropriate suspension delivered aseptically from a sterilized shielded burette. The use of sterile pipettes for inoculating 300–400 flasks proved tiring and also increased the chances of contamination by constant opening of the inoculum tube and decreasing steadiness of hand. An inoculating burette was therefore designed; it could be sterilized, filled aseptically and made to deliver the inoculum quickly and accurately. The burette and the method of filling it are illustrated in Fig. 1.

A glass funnel *A* was blown on to the stem of an ordinary chemical burette so that the jet was adequately shielded. The burette was cut off at the 25 ml. level and the end was plugged with cotton-wool and fitted with a rubber and glass connexion *B* to which could be attached the mouthpiece *C* for filling by suction. For autoclaving, the funnel *A* was plugged with cotton-wool and the whole burette fitted into a copper cylinder. Convenient lengths of glass tubing with rubber connexions were autoclaved, separately wrapped or in a plugged boiling tube; when the burette was to be filled, the rubber end of one of these, *D*, was fitted aseptically on to the jet and the glass end flamed and dipped into the suspension, *E*. The mouth of each flask to be inoculated was flamed and held within the shield immediately below the jet, the inoculum being then run in, the mouth of the flask reflamed and the plug replaced. The bacteria remained evenly distributed through the suspension during delivery of 25 ml. in 0·5 ml. amounts. After a little practice in manipulation, preparing the suspensions, filling the burette and inoculating the flasks could all be done by one junior operator. Two burettes were made and used, one for biotin assays and the other for filling first with the weaker suspensions for assay of pantothenic acid. After being rinsed with sterile water—filling by suction and emptying by gentle blowing through the mouthpiece—it was then filled with the stronger suspension to inoculate flasks for assay of nicotinic acid. Used on an open bench in a room by no means bacteriologically perfect but reasonably free of draughts, these burettes have maintained aseptic delivery for more than 2 h.

Incubation of all assay and standard flasks for 72 h, at 30° for assays of biotin and pantothenic acid and at 37° for nicotinic acid, was found to give most consistent results. Observations made here on the effect of temperature differences within the incubator agreed with those of Price & Graves (1944). Differences between shelves in the incubators used were very slight: from side to side and back to front on the same shelf they were negligible. Each shelf could accommodate seventy-five flasks, i.e. a series of twenty-one flasks for the standard curve and six sets of nine flasks each, for assay of the vitamin in six extracts. Each basket of the autoclave used could also accommodate this number. When more than seventy-five flasks were required for assay of one vitamin, one out of each group of triplicates was incubated on a second shelf and the mean response in each three was taken as the reading. All the flasks used in the assay of the same vitamin were of the same type of glass and had the same base area. They did not require to be shaken during incubation.

Response of the organism in assay of each vitamin was measured by direct titration of the lactic acid produced against sodium-hydroxide solution (approx. 0·1 N) with bromothymol blue as internal indicator. This method of comparing the response in standard and assay flasks appeared more reliable than comparison by estimations of growth based on turbidity measurement. McIlwain & Stanley (1948) have shown that the production of lactic acid is proportional to the dose of nicotinic acid, whereas the bacterial growth measured turbidimetrically is not.

All standard and assay flasks involved in the estimation of any one vitamin were removed from the incubator together; all were titrated to the same end-point (pH about 6·8), which was measured visually, the last flask being titrated as soon as possible after the first. The means of the triplicate readings at each level of pure vitamin or test preparation were plotted to give 'standard' and 'test-preparation' curves. Close agreement between titrations of replicates was usually found. Table 2 shows the results of some typical assays.

RESULTS

Typical standard and test-preparation curves obtained in assays of nicotinic acid, pantothenic acid and biotin are shown in Figs. 2–4. Both curves in nicotinic-acid assays were linear over the range of the vitamin assayed. The slope-ratio method of computation (Wood, 1945, 1946) could thus be used for calculating the results, which agreed closely with those obtained by direct reading from the standard curve. In assays of biotin and pantothenic acid the single-curve method was used, there being no true linear part of the curve. In both instances smooth curves were readily obtained; with the inclusion of *p*-aminobenzoic acid in the medium, responses to amounts of pantothenic acid up to 0·04 μg (occasionally 0·06 μg) were sometimes linear, though not consistently so. With a blank of about 1·0 ml., omission of *p*-aminobenzoic acid gave titrations of 5–7 ml. of approx. 0·1 N-NaOH for 0·08 μg pantothenic acid, whereas including it gave titrations of 10–12 ml. Portions of test preparation were chosen to give readings between 0·01 and 0·06 μg pantothenic acid and 0·00005 and 0·0002 μg DL-biotin. When test materials proved unexpectedly potent or weak, assays were repeated to give readings falling within these limits. Concordance was good, results on a single extract at different levels of assay usually agreeing within ± 5% (Table 2).

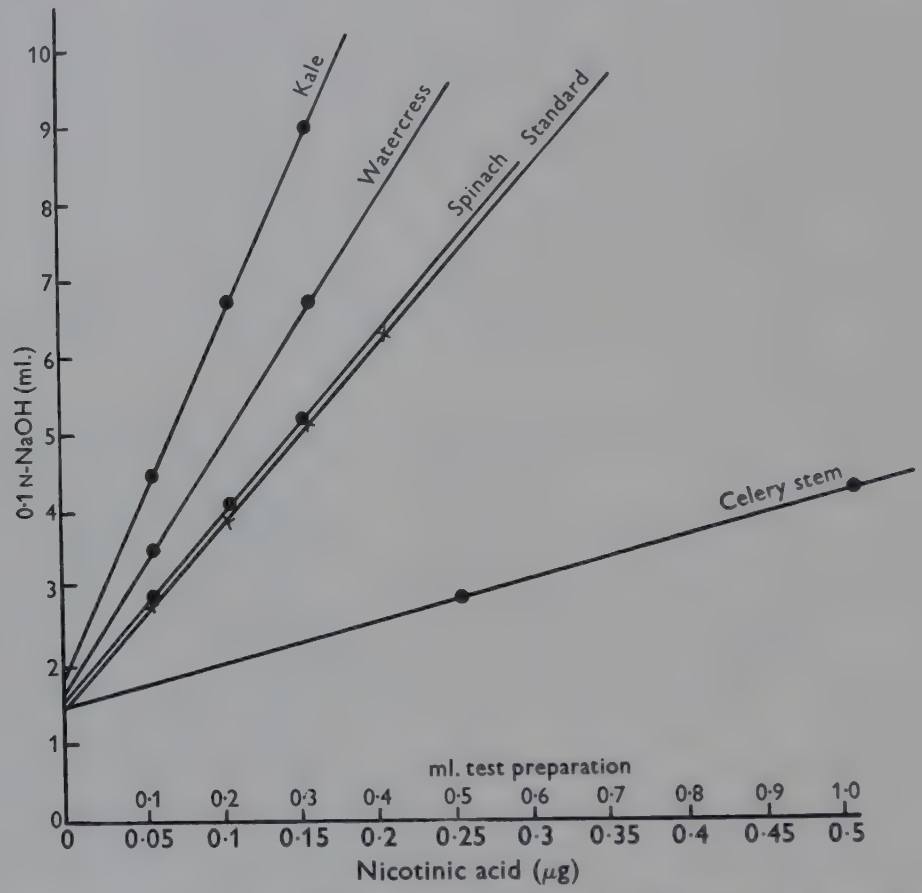

Fig. 2. Nicotinic acid in vegetables. Response of *Lb. arabinosus* to a 'standard' solution of nicotinic acid and to test extracts.

The nicotinic-acid, pantothenic-acid and biotin contents of a wide range of assayed fresh fruits, vegetables and nuts are given in Tables 3–5. Varieties giving outstandingly high or low values are mentioned separately and these figures are not included in the mean.

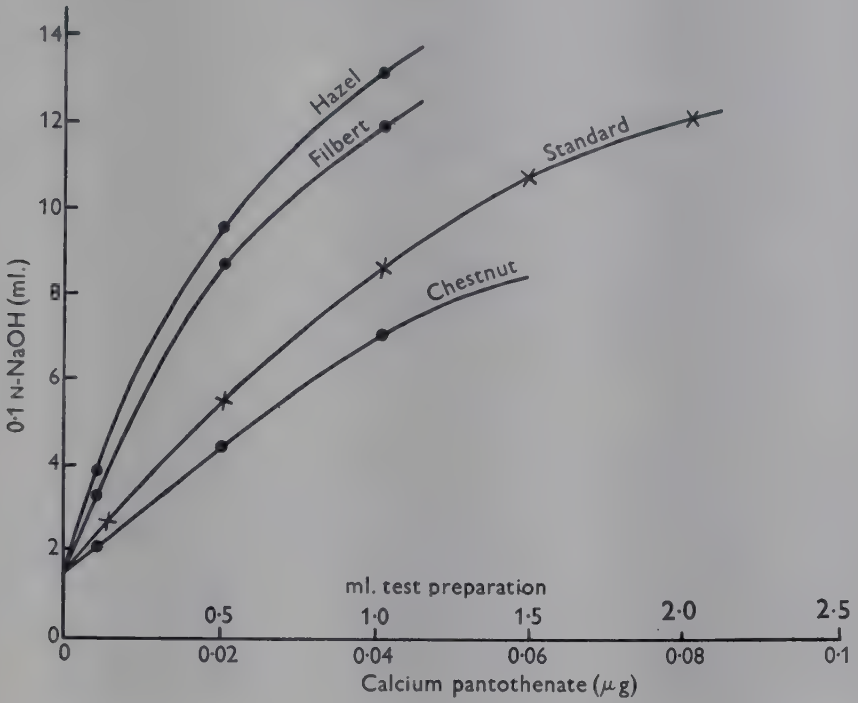

Fig. 3. Pantothenic acid in nuts. Response of *Lb. arabinosus* to a 'standard' solution of calcium pantothenate and to test extracts.

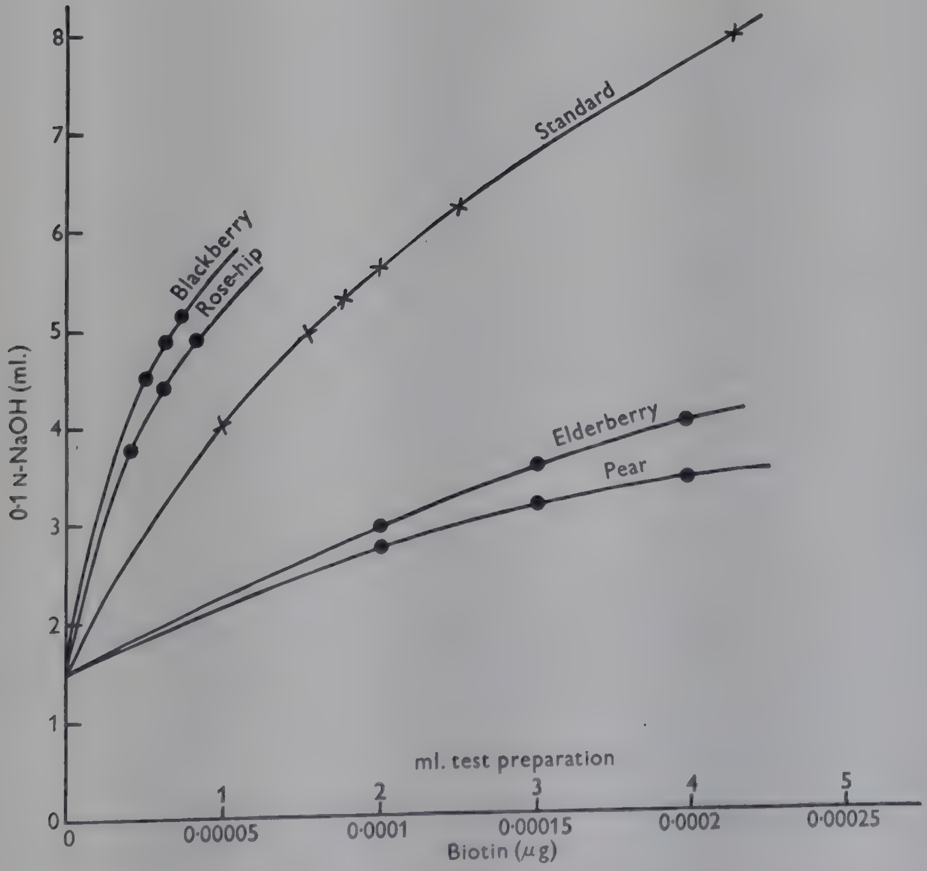

Fig. 4. Biotin in fruits. Response of *Lb. arabinosus* to a 'standard' solution of biotin and to test extracts.

Although the method of estimation gave results well within the ± 10% variation commonly allowed in microbiological assays, the number of samples involved varied from twelve for tomatoes to two for asparagus, being intentionally limited so that as wide as possible a range of species could be covered. These values should therefore be regarded only as indicating the general levels of nicotinic acid, pantothenic acid and biotin to be expected in foods of this kind.

Variations due to season or site may occur in the same variety, and this possibility has not been fully investigated. That such variations may be slight is, however, suggested by results in another experiment in which it was necessary to analyse leaves of healthy immature cauliflowers of the same age from sites more than 100 miles apart. Values obtained for site 1 were: nicotinic acid 1150 μg, pantothenic acid 500 μg and biotin 0·16 μg/100 g leaf respectively, and for site 2, 1100, 580 and 0·37 μg/100 g leaf, respectively. Blackberries and elderberries also gave similar values irrespective of site. Differences between greenhouse and garden tomatoes were insignificant, as were those between parthenocarpic (induced by hormone treatment) and normal fruits. No seasonal variation was observed in a limited number of samples of tomatoes and apples picked from the same sites in 1947 and 1948. No explanation can be offered for the different values shown by plum varieties; all were grown under similar conditions and were picked and assayed at similar stages of maturity. Differences in contents of soluble solids and dry-weight ratios are too small to afford an explanation, and the inclusion in assays of wild yeasts present in surface 'bloom' cannot be considered, as the fruits were thoroughly washed and wiped before extraction. A wide difference was observed between samples of washed and unwashed elderberries, values for nicotinic acid in the unwashed, though apparently clean, fruits being 1800 μg/100 g, whereas the washed fruit contained 600 μg/100 g. This loss on washing may have been due partly to removal of wild yeasts and also to leaching.

The nicotinic-acid and pantothenic-acid contents of whole turnips were higher than those of peeled turnips, averaging for whole turnips 800 and 260 μg, respectively. There was, however, no difference between whole potatoes and those peeled or scraped before assay. The fruits examined contained nicotinic acid in amounts ranging from 50 μg/100 g in Bramley's Seedling apples to 500 μg in tomatoes and 600 μg in elderberries. Pantothenic acid varied from 40 μg/100 g in Cox's Orange Pippin to 400 μg in black currants and rose-hips (apple again being lowest). Biotin was present in fruits in very much smaller amounts, black and red currants with 2·6 μg/100 g having most; plums had only a trace.

Vegetables, in general, were found to contain higher proportions of these three vitamins than fruits, but again the range was wide. Broad beans and peas were outstandingly high in both nicotinic and pantothenic acids, the former also having the highest biotin content of all the vegetables examined. Parsley (included among the vegetables owing to its frequent use in salads and sauces and its comparatively high content of ascorbic acid and carotene), also was high in nicotinic acid but unexpectedly low in pantothenic. Cauliflower leaves were far richer in nicotinic acid than the flower and stalk, but weight for weight the flower contained more pantothenic acid and biotin.

Table 2. *Examples of assay results*

Test material	Weight taken (g)	Dilution	Volume assayed (ml.)	Titration approx. 0·1 N-NaOH (ml.)	Mean titration (ml.)	Vitamin content	
						a, slope ratio* *b* and *c*, µg/flask	µg/100 g†
Walnut, small variety	2 (defatted, fat=59% of peeled kernel)	*a*, × 100	0·1	2·9, 3·2, 3·3	3·1	$\frac{7·5}{2·13}$	1443
			0·3	4·8, 4·6, 4·5	4·6		
			0·5	5·7, 5·8, 6·2	5·9		
		b, × 100	0·1	3·0, 3·2, 3·3	3·2	0·008	328
			0·5	8·9, 8·5, 8·7	8·7	0·0405	332
			1·0	12·4, 11·8, 11·7	12·0	0·0802	329
		c, × 1000	1·0	3·0, 3·1, 3·1	3·1	0·0001	2·05
			1·5	3·5, 3·5, 3·6	3·5	0·000147	2·0
			2·0	4·2, 4·0, 4·1	4·1	0·000212	2·15
Tomato, outdoor variety	50	*a*, × 40	0·5	4·3, 4·3, 4·2	4·3	$\frac{5·06}{2·99}$	677
			0·6	4·8, 4·8, 4·7	4·8		
			0·7	5·4, 5·2, 5·3	5·3		
		b, × 40	0·8	6·0, 6·3, 5·9	6·1	0·0167	84
			1·0	7·0, 7·1, 6·9	7·0	0·0211	84
			1·5	8·4, 8·4, 8·3	8·4	0·032	85
		c, × 100	0·2	2·5, 2·5, 2·5	2·5	0·000054	1·35
			0·4	3·2, 3·4, 3·4	3·3	0·000113	1·41
			0·6	4·1, 4·1, 4·1	4·1	0·000173	1·44
Runner bean, Scarlet Empress	10	*a*, × 20	0·1	2·6, 2·4, 2·5	2·5	$\frac{8·15}{1·72}$	948
			0·2	3·3, 3·3, 3·2	3·3		
			0·3	4·2, 4·0, 4·1	4·1		
		b, × 20	0·1	3·4, 3·4, 3·4	3·4	0·002	40
			0·2	3·9, 3·9, 4·0	3·9	0·0045	45
			0·3	4·6, 4·7, 4·5	4·6	0·007	46
		c, × 20	0·1	3·5, 3·6, 3·3	3·5	0·000068	0·68
			0·2	4·6, 4·8, 4·7	4·7	0·000143	0·71
			0·3	5·5, 5·4, 6·0	5·6	0·000214	0·71

a=nicotinic acid, *b*=pantothenic acid, *c*=biotin.

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* Slope ratio: $\frac{\text{response to test preparation (ml. NaOH/ml.)}}{\text{response to `standard' (ml. NaOH/0.1}\mu\text{g)}}$.

† The biotin used in stock and standard solutions was all DL-biotin supplied by Ashe Laboratories Ltd. The response of the organism to DL-biotin is half that to D-biotin, which is the natural form and to which the word 'biotin' usually refers. All results based on DL-biotin standard curves (col. 7) had therefore to be halved to give correct biotin values (col. 8).

With the exception of pulse vegetables no class of fruits or vegetables appeared to be consistently richer than another in any one of these vitamins. For example, root vegetables included beetroot with a nicotinic-acid content of 110 μg/100 g, and swedes and parsnips with more than 1000 μg/100 g. Hard fruits did, however, tend to be low in pantothenic-acid content. Some kinds of nuts contained far greater proportions of biotin than fruits or vegetables. Filberts and hazel nuts were outstanding in this respect, whereas almonds were surprisingly low in both biotin and pantothenic acid, but highest of all nuts examined in nicotinic acid. Chestnuts were consistently low in all three vitamins.

Table 3. *Nicotinic-acid, pantothenic-acid and biotin contents of fresh fruits*

Fruit		No. of varieties	No. of samples	Nicotinic acid (μg/100 g)		Pantothenic acid (μg/100 g)		Biotin (μg/100 g)	
				Range	Mean	Range	Mean	Range	Mean
Apple:									
Allington	(*a*)	1	2	—	77	—	49	—	0·27
Pippin	(*b*)	1	2	—	—	—	—	—	0·48
Cox's Orange	(*a*)	1	2	—	94	—	43	—	0·26
Pippin	(*b*)	1	2	—	—	—	—	—	0·65
Bramley's	(*a*)	1	2	—	50	—	49	—	0·24
Seedling	(*b*)	1	2	—	—	—	—	—	0·42
Edward VII	(*a*)	1	2	—	85	—	70	—	0·24
	(*b*)	1	2	—	—	—	—	—	0·23
Blackberry (wild)		2	4	229–414	350	200–249	224	0·36–0·52	0·43
Black currant		2	4	233–293	262	389–403	398	2·03–2·7	2·4
Cherry: Morella		1	2	—	323	—	73	—	0·96
sweet		1	2	—	173	—	81	—	0·38
Damson		1	2	—	253	—	266	—	0·12
Elderberry (wild)		3	4 (washed)	570–620	600	102–118	110	0·029–0·06	0·042
			2 (unwashed)	1761–1834	1798	128–136	132	—	0·1
Gooseberry: culinary		2	4	130–389	260	135–145	140	0·35–0·67	0·47
dessert		1	4	243–260	250	257–302	286	—	0·07
Pear: Bristol Cross and Conference		2	4	170–217	183	36–56	43	0·055–0·085	0·07
William		1	2	—	257	—	50	—	0·079
Plum: Victoria and Teme Cross		2	4	160–171	166	116–141	129	Nil–0·035	Trace
Monarch		1	2	—	540	—	239	—	0·07
Frome Cross		1	2	—	434	—	160	—	0·045
Raspberry		2	4	329–429	380	160–268	211	1·71–2·08	1·87
Red currant		1	2	—	130	—	64	—	2·63
Rose-hip, wild		2	2	—	420	—	393	—	0·32
Strawberry		2	4	—	380	—	83	1·01–1·25	1·11
Tomato: indoor		2	4	—	518	19–66	42	1·33–1·53	1·44
outdoor		4	8	—	539	38–81	54	0·55–1·4	1·0

(*a*) 1 week after picking. (*b*) 12 weeks after picking.

When assessing the nutritional value of any food, consideration must be given to the fact that the state in which a food is analysed is not always that in which it is consumed. Although in the foods concerned here extraction was always made of the fresh raw material, heating formed an essential part of the extraction process. Thus all values found are virtually of the cooked food, and the possibility of the presence of such systems as the avidin-biotin complex in the raw material must not be overlooked. As these assays were made on enzyme-digested extracts, the values obtained for nicotinic acid may reflect the presence of a nicotinic-acid precursor. The precursor in wheat and other biological materials has been shown (Krehl, Elvehjem & Strong, 1944) to be

Table 4. *Nicotinic-acid, pantothenic-acid and biotin contents of fresh vegetables*

Vegetable	No. of samples	Nicotinic acid (μg/100 g)	Pantothenic acid (μg/100 g)	Biotin (μg/100 g)
Asparagus: 'choice'	1	1000	174	0·58
'selected'	1	857	187	0·55
Bean: broad	2	4250	5100, 5625	3·22
dwarf	2	376, 490	100	1·17, 1·36
runner	2	939	45, 51	0·7
Beetroot	2	100, 120	133, 158	0·02, 0·045
Brussels sprouts	2	560, 760	320, 395	0·43
Cabbage, savoy	2	234	183	0·08
Carrot, young	2	812	350	0·59
Cauliflower: flower	2	567	642	1·9
leaf	2	1400	413, 470	0·44
stalk*	2	458	217	0·14
Celery: stem	2	250	375	0·13
leaf and midrib*	2	680	640	0·5
Kale, curly	2	1045	350	0·46
Lettuce, cabbage	2	319	44	0·62, 0·83
Leek	2	588, 747	120	1·38
Marrow	1	265	103	0·42
Onion	2	175	89	0·9
Parsley	2	821	25	0·43
Parsnip	2	980, 1300	400	0·108
Pea	2	1913, 2350	1450, 1800	0·43, 0·66
Potato, early main crop (November):				
scraped	2	400	160	0·135
peeled	2	426	300	0·098
whole	2	492	320	0·12
Spinach, perpetual	2	500	345	0·15
Swede	2	1108	109	0·08
Turnip: peeled	2	281, 342	18	0·11
whole	2	750, 827	26	0·053
Watercress	2	542, 644	70	0·4

* All other values refer to edible parts only.

Where results on two samples differed by more than 10%, both are shown.

Table 5. *Nicotinic-acid, pantothenic-acid and biotin contents of home-grown nuts*

Nut	Nicotinic acid (μg/100 g)	Pantothenic acid (μg/100 g)	Biotin (μg/100 g)	Fat (%)
Almond*	1670	75	0·4	45
Chestnut*	206	227	1·3	17·5
Filbert*	500	465	14·3	55
Hazel: peeled kernel	600	380	15·8	58
whole kernel	715	480	17·3	62
Walnut: small*	1440	349	2·0	59
large (Northdown clawnut)*	780	600	1·8	48
Sunflower seed*	5040	620	8·7	55

* Peeled kernel.

With the exception of walnuts, assays were made on a bulk sample of each kind from two or more sources.

biologically active for days, and as suggested by Sohonie & Misra (1950) it may be reasonable to suppose that values for nicotinic acid obtained by enzymic extraction represent what is biologically available to man. Check by biological assay is thus indicated.

DISCUSSION

It is interesting to note that the vegetables containing most nitrogen, namely the pulses, were found to contain most nicotinic acid. Also almonds and walnuts, which have comparatively high protein contents, contained considerably more nicotinic acid than the starchy chestnut.

The vegetables known to contain most nitrogen were also found to contain most pantothenic acid. Lipmann, Kaplan, Novelli, Tuttle & Guirard (1947) have shown coenzyme A to be a derivative of pantothenic acid, and Kaplan & Lipmann (1948) have estimated that tomatoes contain nearly twice as much coenzyme A as spinach. Here the perpetual spinach assayed contained considerably more pantothenic acid than tomatoes, indicating no correlation between coenzyme A and pantothenic-acid contents.

Evidence has been provided (Axelrod, Purvis & Hofmann, 1948) that biotin forms part of an enzymically active complex, and investigation of biotin activation of deaminases (Lichstein & Umbreit, 1947*b*; Lichstein & Christman, 1948) suggests the possibility of a biotin-adenylic acid combination in a structure similar to the di- and tri-phosphopyridine nucleotides of nicotinic acid. Its relationships to the synthesis of oleic acid (Williams & Fieger, 1946; Williams, Broquist & Snell, 1947) and to oxalacetic acid decarboxylation (Lichstein & Umbreit, 1947*a*; Lardy, Potter & Elvehjem, 1947; Shive & Rogers, 1947; Ochoa, Mehler, Blanchard, Jukes, Hoffmann & Regan, 1947) have been indicated and suggest that biotin may be the coenzyme of oxalacetate decarboxylase. More recent work of Lardy, Potter & Burris (1949) points to an essential role in the metabolic fixation of carbon dioxide.

Even against so incomplete a background the biotin values obtained in fruits are suggestive. With the exception of wild blackberries and elderberries, the soft fruits contained a greater amount of biotin than the hard and stone fruits. Such fruits as these, strawberries, raspberries, red currants, have a short 'mature life', which may be correlated with a high rate of cell respiration. The two exceptions may be discounted as having a much lower proportion of flesh and juice to seed than the other soft fruits examined.

Again, the four varieties of apple tested showed very similar biotin values (0·24–0·27 μg/100 g) when assayed within a week of picking in a thoroughly unripe condition. Apples from the same batches assayed 12 weeks later differed both in maturity and in biotin content. Samples of Cox's Orange Pippin were perfectly ripe (biotin 0·65 μg/100 g) and those of Allington Pippin (0·48 μg) and Bramley's Seedling (0·42 μg) were semi-ripe. Samples of Edward VII were still hard and green, there being no apparent change in maturity and no increase in the biotin content.

The apparent relationship between biotin content and maturity would appear to merit further investigation. It may also serve to explain the wide variations found in the biotin contents of nuts.

SUMMARY

1. Nicotinic acid, pantothenic acid and biotin have been estimated microbiologically in a wide range of fruits, vegetables and nuts commonly grown in the British Isles.

2. Microbiological methods of assay were used with *Lactobacillus arabinosus* as test organism. They proved suited to the simultaneous estimation of nicotinic acid, pantothenic acid and biotin in a single enzyme-extracted preparation of test material. The procedure followed is fully described and examples of assay results and response curves are given.

3. In fruits, amounts of nicotinic acid varied from 50 to 90 μg/100 g in apples to 260 μg/100 g in gooseberries and black currants, and 520 μg/100 g in tomatoes. Pantothenic-acid values ranged from 40 to 70 μg/100 g in apples, pears and tomatoes to nearly 400 μg/100 g in rose-hips and black currants. Biotin was found in small quantities, from a trace in plums to 1·0 μg/100 g in tomatoes and about 2·5 μg/100 g in red and black currants.

4. In vegetables, nicotinic-acid levels were mostly higher than in fruits. Pulse vegetables were markedly the highest in all three vitamins, broad beans containing nicotinic acid 4250 μg/100 g, pantothenic acid 5000 μg/100 g and biotin 3 μg/100 g. Other vegetables varied in nicotinic-acid content from 175 μg/100 g in onion to 400–500 μg/100 g in potato, and 1000 μg/100 g in curly kale, parsnip and asparagus. Pantothenic-acid values were found from 18 μg/100 g in turnip to 350 μg/100 g in curly kale and carrot and 640 μg/100 g in cauliflower. Biotin contents ranged from <0·1 μg/100 g in beetroot, turnip and swede to nearly 0·5 μg/100 g in brussels sprouts, parsley, watercress and curly kale, and 1·9 μg/100 g in cauliflower.

5. In nuts, amounts of nicotinic acid varied from 200 μg/100 g in chestnuts to 600 μg/100 g in hazel and 1600 μg/100 g in almond; 5000 μg/100 g were found in sunflower seeds. Pantothenic-acid values ranged from 75 μg/100 g in almond to 380 μg/100 g in hazel and 600 μg/100 g in walnut; 620 μg/100 g were found in sunflower seeds. Biotin was present in greater quantities than in fruits and vegetables, varying from 0·4 μg/100 g in almond to 2 μg/100 g in walnut and 14–16 μg/100 g in filbert and hazel; 8·7 μg/100 g were found in sunflower seeds.

The author is indebted to Miss E. M. Glenn of East Malling Research Station for supplying the walnut varieties and to Mr Cyril Vint for the samples of asparagus. Thanks are also due to Miss Crang and her assistants for help with the experimental work.

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Factors Affecting the Utilization of Food by Dairy Cows

5. The Digestibility and Rate of Passage of Foods during L-Thyroxine Administration

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(*Received* 29 *August* 1951)

It is well known that the administration of thyroid-active substances to lactating cows usually stimulates milk secretion and, in this way, may affect the efficiency of food utilization. Until recently iodinated protein was the cheapest and most suitable thyroid-active substance available, but synthetically produced L-thyroxine, given by mouth, now appears to be the most suitable material (Bailey, Bartlett & Folley, 1949). The administration of thyroid-active substances raises the metabolic rate and increases the nutritive requirements of cows. The literature on this subject has been reviewed by Blaxter, Reineke, Crampton & Petersen (1949), and further results have been reported by Moustgaard & Thorbek (1949). The experiment described in this paper was an attempt to find whether this increased requirement is met, even partly, by a more efficient digestion of available food or whether the cows can obtain additional nutrients only by eating more food or utilizing body reserves.

The administration of thyroid-active materials is known to increase the rate of stomach emptying and intestinal motility in non-ruminants (see review by Ivy, 1930). In cows the administration of iodinated protein (Blaxter, 1946) and of thyroxine (Owen, 1948) is known to produce moister faeces, and the same effect has been observed in sheep given heavy doses of iodinated casein (Blaxter, 1948); this has sometimes been thought to signify a faster rate of passage of food. The effect of an increased rate of passage on digestive efficiency would depend on simultaneous changes in the rate of production of the digestive secretions and on the rate of absorption of the products of digestion; if these rates were also increased, the digestibility of the food might not be affected. It was shown by Fink (1944) that thyroid extracts cause an increased production of digestive secretions in the jejunum of the dog, and Althausen & Stockholm (1938) found that in the rat thyroidectomy decreases dextrose absorption in the digestive tract and that the administration of thyroxine increases the rate of absorption of dextrose, galactose, xylose and oleic acid but not of alanine. Blaxter (1948) found that heavy doses of iodinated casein depress the digestibility of the dry matter and crude protein of mixed diets of hay, oats and flaked maize in sheep fed according to standard (Kellner, 1907). Owen (1948) found an increased faecal excretion of nitrogen during the administration of thyroxine to cows on a low plane of nutrition, but not to cows on a high plane of nutrition, and tentatively suggested that the increased excretion of nitrogen might be due partly to a more rapid rate of passage preventing optimum absorption. Results are here reported of an experiment in which the effect of L-thyroxine on digestibility and rate of passage was determined in lactating cows receiving an ample diet.

METHODS

Cows and their treatment. For the experiment, which lasted 10 weeks and was divided into two main parts, four Shorthorn cows in declining lactation were selected from the Institute herd.

The cows were given the same diet of hay and concentrates throughout, but during the second part of the experiment, which included the 5th–10th weeks, they were each given orally 100 mg L-thyroxine/day. (This was later reduced in two cows, see p. 358.) During the 1st week the intake of hay was adjusted to a level almost sufficient to satisfy appetite, and concentrates were fed at the rate of 0·4 kg/kg milk produced in the previous week. In the 2nd week the cows were fed the same amount of hay, and concentrates at the rate of 0·4 kg/kg milk produced in the 1st week plus an additional 1·8 kg concentrates/cow/day. These amounts were given throughout the remainder of the experiment (Table 1). The hay was of a coarse type containing little clover, and with Italian ryegrass (*Lolium italicum*) predominating. The concentrate mixture contained dredge corn (oats and beans) 30, wheat bran 10, coconut meal 20, palm-kernel cake 20, decorticated cottonseed cake 10, and decorticated groundnut cake 10, parts with the addition of 2% of a commercial mineral mixture. Each animal had access to a salt lick. These diets proved ample to satisfy appetite, but food was refused on a few occasions.

Table 1. *Foods offered to cows in weeks 2–10 inclusive*

Cow	Hay (kg/day)	Concentrates (kg/day)
A	9·1	7·3
B	7·3	7·3
C	5·4	7·8
D	9·1	8·2

Thyroxine administration. During the 5th week all the cows were given L-thyroxine*. The dose was successively 25, 50, 75 and 100 mg on the first 4 days of the week and continued at 100 mg daily until the 8th week when it became obvious that this was above the optimum dose for cows B and C, which were showing signs of slight distress; they had very high heart rates and were restless. These cows were accordingly given 75 mg/day in the 8th week and subsequently. In the 10th week all the doses were gradually reduced to nil.

Measurements of the digestibility and rate of passage of the food. Measurements of the digestibility of the diet and of the rate of passage of hay through the digestive tract were made in two collection periods of 14 days. These periods occupied weeks 3–4 and 8–9 during the control and thyroxine treatments respectively. By arranging the treatments in this way the results were not affected by any after-effects of thyroxine treatment.

The digestibility trials and measurements of the rate of passage of hay were essentially similar to those described by Balch (1950) for collection periods of 10 days. Faeces were collected in rubber bags supported on a harness worn by the cows, and separated from urine by a special separating device also fixed to the harness (Balch, Bartlett & Johnson, 1951). By using a 14-day collection period it was possible to calculate digestibility coefficients in each of the 2 weeks, and this gave an indication of the accuracy of the method. The longer period also made possible a check of the accuracy of the determinations of the rate of passage of hay. This was done by giving small meals of hay stained with magenta on the 1st day, and of hay stained with brilliant green on the 4th day, of each collection period. The method of sampling faeces, counting stained particles in the samples and plotting excretion curves was described earlier by Balch (1950).

Other measurements. Throughout the experiment, records of the amount of water drunk by the cows, of their heart rates and rectal temperatures were made daily, and the cows were weighed at intervals.

The cows were milked twice daily by machine. Twice weekly, determinations of the content of total solids, by the gravimetric method, fat by the Gerber method and solids-not-fat by difference, were made on samples of milk from four consecutive milkings bulked in proportion to the yield at those milkings.

RESULTS

Effect of thyroxine. Measurement of the heart rate, rectal temperature and of the amount of water drunk showed clearly that the daily administration of 100 mg thyroxine per cow produced a marked rise in metabolism (Fig. 1). The heart rate of every cow increased by 10–20 beats/min and began to fall as the dose of thyroxine was reduced. Three of the four cows drank more water during thyroxine administration. Fig. 1 also shows that the rate of decline in milk yield was altered in all the cows. The yield rose for 2 weeks before falling at about the same rate of decline as in the control period. During the control period the weight of the cows changed little, apart from that of cow A, which rose, but while thyroxine was being given all the cows lost weight, the weekly losses varying from 2·4 to 8·3 kg/cow (Table 2).

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* As cubes containing 6 g oatmeal and 25 mg monosodium L-thyroxine.

Digestibility coefficients. The mean digestibility coefficients in the 14-day collection periods are given in Table 3. There were small differences between the coefficients for individual cows, due in part to variations in the proportion of hay and concentrates given to the cows. However, in any one cow the differences between values for different weeks of the same collection period were almost invariably considerably greater than the differences between the mean values for the two whole collection

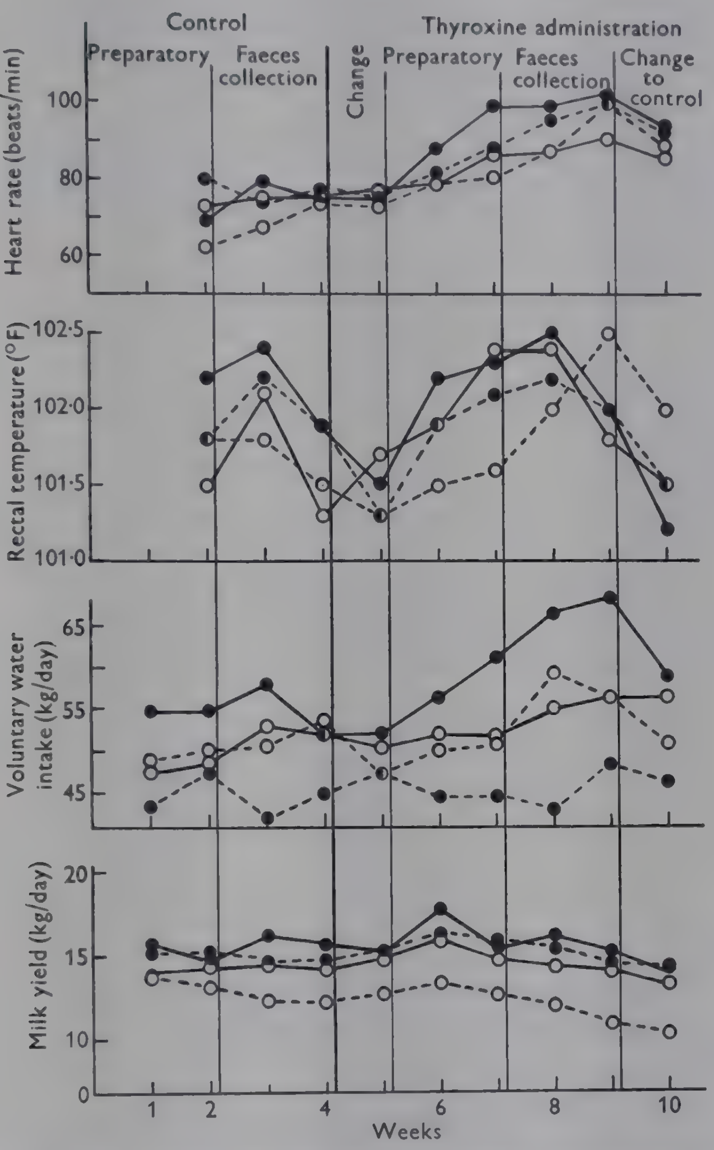

Fig. 1. Effect of thyroxine administration on the heart rate, rectal temperature, voluntary water intake, and milk yield of cows. Weekly mean values of daily observations are shown for cows A, ○- - -○; B, ○——○; C, ●- - - -●; and D, ●——●.

Table 2. *Weights of cows (kg)*

Cow	Beginning of experiment	End of part 1 (control)	End of part 2 (thyroxine administration)	Mean weekly change: Control period	Mean weekly change: Thyroxine period
A	617	632	583	+4·3	−2·4
B	499	497	475	−0·6	−4·0
C	495	492	469	−0·9	−4·3
D	623	623	580	0	−8·3

periods and use of the t test showed that the probability of any differences due to treatment being greater than the differences due to week-to-week variation was not significant. The greatest differences between the treatments were observed in the digestibility of crude fibre, but here the variation between different weeks of the same collection period was also greatest.

Table 3. *Effect of daily administration of 100 mg thyroxine/cow on the digestibility of a mixed diet of hay and concentrates*

(Mean values for four cows)

	Digestibility (%)		
Nutrient	Control period	Thyroxine period	Change due to thyroxine
Dry matter	62·8	63·8	+1·0
Crude protein	67·7	68·8	+1·1
Ether extract	82·9	80·9	−2·0
N-free extract	68·1	68·4	+0·3
Crude fibre	51·3	54·3	+3·0

Table 4. *Time* (*h*) *necessary for the first appearance in the faeces of the cows of residues from a meal of stained hay*

	Control period		Thyroxine period	
Cow	Red hay	Green hay	Red hay	Green hay
A	16	16	16	16
B	12	12	12	12
C	16	12	12	12
D	16	16	16	16

Rate of passage of hay through the cow. The time of the initial appearance of stained residues in the faeces was remarkably constant and was unaffected by the administration of thyroxine (Table 4). The first appearance of residues from stained food in the faeces of cows B and C was always observed in the samples of faeces taken 12 h after feeding, with the exception of red hay in cow C during the control period which first appeared 16 h after feeding. Excretion from cows A and D was spread over a longer period than excretion from cows C and B (Table 4), and residues from both coloured hays were invariably first detected in samples of faeces which accumulated in the period 12–16 h after feeding.

Details of the further progress of excretion of undigested stained hay particles are shown by excretion curves in Fig. 2. The time required for excretion of 5% of the total stained particles excreted in 240 h, which will be called the 5% excretion time, varied more than the time of the initial appearance of the particles in the faeces (Table 5). The differences suggest that the 5% excretion time was less when the cows were receiving thyroxine, but it is unlikely that they were significant. It has been shown (Balch, 1950) that the first appearance of stained foods in the faeces, whether actually determined, as in Table 4, or judged by the time required for the excretion of the first 5% of the total undigested particles, as in Table 5, is indicative of the time required for the excretion of food that has left the reticulo-rumen. Therefore, it can be concluded that the administration of thyroxine to milking cows did not markedly change the total time required for food residues to pass from the reticulo-rumen to the faeces.

Table 5. *Time (h) required for excretion by the cows of undigested residues from stained hay*

Period	Proportion of total residues from stained hay (%)	Cow A		Cow B		Cow C		Cow D	
		Red hay	Green hay	Red hay	Green hay	Red hay	Green hay	Red hay	Green hay
Control	5*	29	29	21	21	30	30	24	22
	80*	107	124	91	100	138	126	101	90
	80 − 5*	78	95	70	79	108	96	77	68
Thyroxine	5*	23	24	21	21	26	30	21	21
	80*	90	100	93	100	126	117	82	86
	80 − 5*	67	76	72	79	100	87	61	65

* For explanation of these indices of the rate of excretion, see pp. 360, 361.

Table 6. *Effect of* L-*thyroxine on the dry-matter content of the total intake and of the faeces of the cows*

Cow	Intake (%)*			Faeces (%)		
	Control period	Thyroxine period	Change due to thyroxine	Control period	Thyroxine period	Change due to thyroxine
A	20·5	18·3	−2·2	17·2	16·2	−1·0
B	18·7	17·6	−1·1	15·4	14·9	−0·5
C	20·1	19·2	−0·9	18·4	16·8	−1·6
D	20·8	17·5	−3·3	15·4	14·4	−1·0
Mean	20·0	18·1	−1·9	16·6	15·6	−1·0

* $$\frac{\text{Dry-matter intake (kg)} + \text{water intake (kg)}}{\text{Water intake (kg)}} \times 100.$$

The moisture content of faeces has sometimes been used as an indication of the rate of passage of food. If this were justified it would probably apply particularly to the rate of passage of food residues through the intestine. In the present experiment the administration of thyroxine caused a decrease in the percentage of dry matter in the faeces of every cow (Table 6). On average the dry-matter content of the faeces fell from 16·6 to 15·6%. This decrease was presumably linked to the raised intake of water during thyroxine administration (Fig. 1) and the resultant fall in the dry-matter content of the total intake (Table 6) but, as has been shown above, it was not accompanied by any change in the index of the rate of passage of food from the reticulo-rumen to the faeces.

The spread of excretion curves, of the type shown in Fig. 2, is indicative of the rate of passage of foods through the reticulo-rumen, and an index of this spread is obtained by taking the difference between the times required for 5 and 80% of the total undigested stained particles to be excreted (Balch, 1950). This will be referred to as the '80 − 5% excretion time'. Values for the spread of the excretion curves are given in Table 5. The mean time required for the excretion of 80 − 5% of undigested

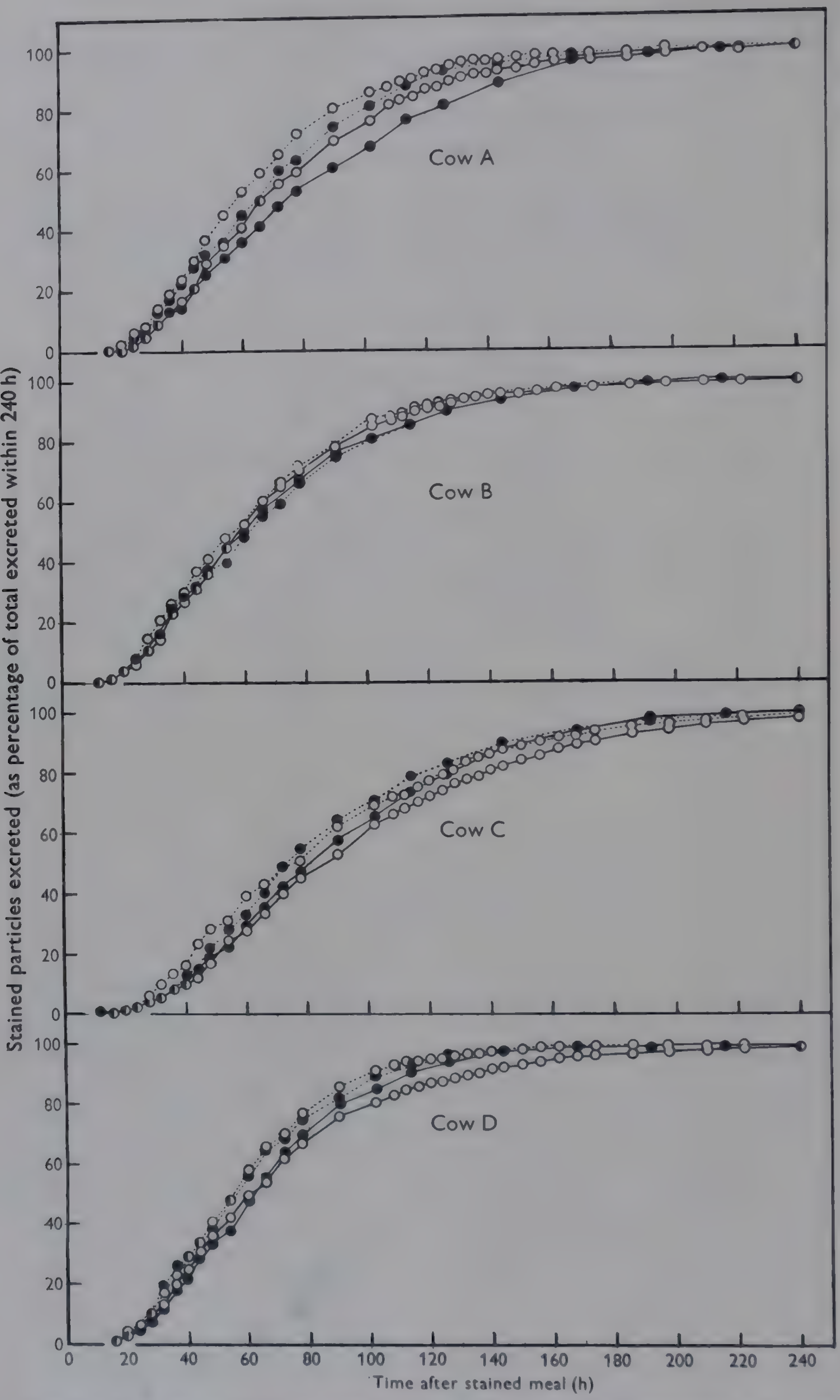

Fig. 2. Values for the excretion of undigested particles (shown as the percentage of the total stained particles excreted within 240 h) of a meal of stained hay in four cows during the control period and period of thyroxine administration. ○——○, control period, hay stained red with magenta; ●——●, control period, hay stained green with brilliant green; ○- - - -○, thyroxine period, hay stained red with magenta; ●- - - -●, thyroxine period, hay stained green with brilliant green.

residues of stained hay was 83·9 h in the control period and 75·9 h when the cows received thyroxine. Use of the *t* test showed that the difference between these means was significantly greater at the 5% level than the difference due to errors in measurement, as shown by the difference between values for hay stained red and green.

It therefore seems that, as a result of the administration of thyroxine, food residues tended to be excreted slightly faster than is normal; the data suggest that this acceleration of rate of passage took place in the reticulo-rumen. There was, however, considerable variation in the extent of the faster excretion between the cows, and in one cow the rate of excretion appeared to be unchanged by thyroxine.

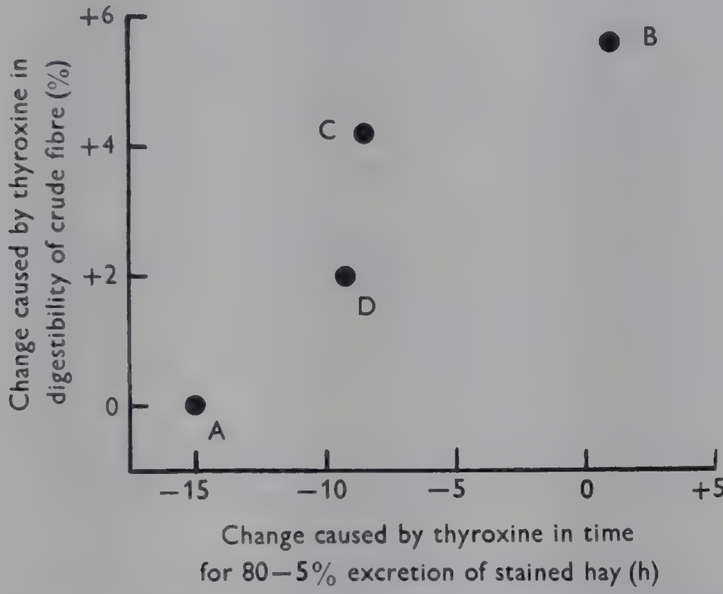

Fig. 3. Relationship between changes in the digestibility of crude fibre and changes in the time required for the excretion of 80−5% of the residues from small meals of stained hay following thyroxine administration to cows A, B, C and D. (For explanation of the 80−5% index of excretion see p. 361.)

Between-cow variations in the changes in the digestibility of crude fibre and the index of the rate of passage of foods through the reticulo-rumen bore a positive relationship (Fig. 3). The significance of this relationship cannot be proved, but it is tempting to suggest that the digestibility of crude fibre was raised in all the cows provided there was not a compensating increase in the rate of passage of the food.

Composition of the milk. Weekly mean values for the composition of the milk of the four cows are given in Fig. 4. In each cow the fat content of the milk rose steadily throughout the 5 weeks of thyroxine administration and dropped as the dose was reduced. The mean solids-not-fat content of the milk increased by 0·1–0·2%. These values are typical of effects of the administration of thyroid-active material to stall-fed cows (Bartlett, Rowland & Thompson, 1949).

DISCUSSION

Changes in the live weight, heart rate, rectal temperature, water intake and milk composition provide ample assurance that the metabolism of the four cows was considerably stimulated during the 5 weeks of thyroxine administration.

The addition of 100 mg thyroxine to the daily concentrate allowance of each cow caused no change in the digestibility of dry matter, or of any of the constituents of the mixed diets of hay and concentrates, nor was there any consistent change in the rate at which the hay passed through the digestive tract.

The effect of thyroxine on the digestibility coefficients for crude protein appears to be in agreement with the work of Owen (1948) who found that the daily intravenous administration of 10 mg thyroxine did not alter the apparent digestibility of dietary nitrogen in amply fed cows, although if the cows were fed at a lower plane of nutrition the apparent digestibility of dietary nitrogen fell. Cows in the present experiment were fed at a level at least as high as the high level of Owen (1948).

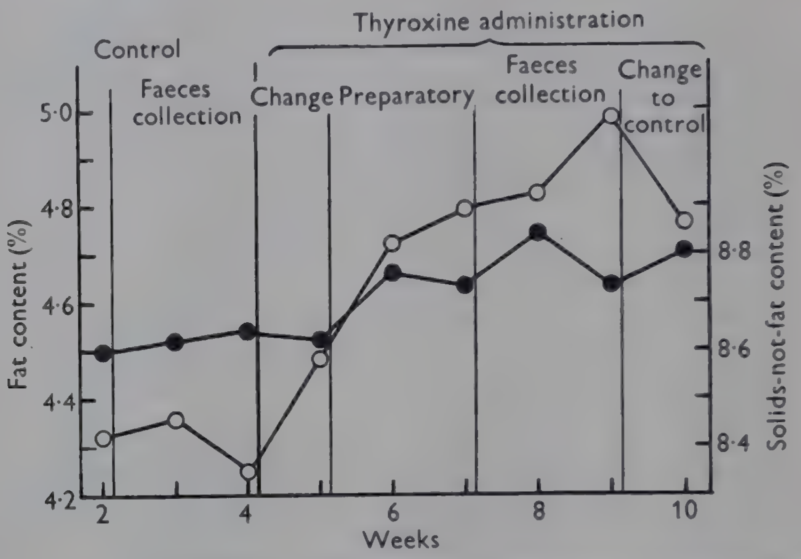

Fig. 4. Effect of thyroxine administration on the fat, ○——○, and solids-not-fat, ●——●, content of milk. Values are weekly means for cows A, B, C and D.

It is probable that the increase in faecal nitrogen and also, therefore, the fall in the apparent digestibility of crude protein, resulting from the administration of thyroid-active substances to cows (Owen, 1948) or sheep (Blaxter, 1948) fed at or below standard requirements, is due to an increase in the metabolic faecal nitrogen resulting from an increased katabolism (Owen, 1948).

Since there was no change in the digestibility of food during thyroxine administration it seems that the cows were depending on surplus food intake or loss of body reserves for their extra nutritional requirements and, in fact, there was some indication that a loss of body-weight occurred even in cows receiving ample food.

The water consumption of cows rose 5–20% during the period of thyroxine administration. This is hardly unexpected in view of the large increases in secretion of urine observed by Owen during thyroxine administration. A possible effect of the rise in water intake was a mean fall in the dry-matter content of the faeces from 16·6 to 15·6%, observed in the present experiment. Owen also observed a fall in the dry-matter content of faeces. It is probable that this rise in water intake slightly lowered the average dry-matter content of the reticulo-ruminal contents. Under such conditions there is reason to expect a more rapid breakdown of fibre in the reticulo-rumen (Balch & Johnson, 1950). No significance can be attached to the positive relationship that appeared to exist during thyroxine administration between the changes in the digestibility of crude fibre and the rate of passage of hay through the reticulo-rumen. It is tentatively suggested, however, that more fluid conditions in the reticulo-rumen raised the digestibility of crude fibre and that in some of the cows there was a simultaneous increase in the rate of passage of foods, which had the opposite effect.

SUMMARY

1. The oral administration of 100 mg L-thyroxine daily to each of four Shorthorn cows caused increases in metabolism evident as increases in the heart rate, body temperature and water consumption and as changes in the composition of the milk.

2. Thyroxine administration caused no significant changes in the digestibility of the dry matter, crude protein, ether-extractable substances, crude fibre or nitrogen-free extract of an ample diet of hay and concentrates.

3. Thyroxine administration caused no consistent change in the rate of passage of foods through the digestive tract. There was evidence, however, that in three of the four cows foods passed slightly more rapidly through the reticulo-rumen, but the rate of passage of food through the remainder of the gut was unchanged.

4. Results suggest that the extra nutritive requirements of cows given thyroxine can be met only by increased food intake or loss of body reserves and not by an increased efficiency of digestion. In spite of ample feeding the cows lost on the average 4·8 kg a week.

5. The voluntary water intake rose during thyroxine administration. The possible effect of this factor and of the small changes in rate of passage is discussed in relation to the digestion of crude fibre in the reticulo-rumen.

Thanks are due to Mr J. Elton for his help with the handling and management of the cows and to Mr A. Wagstaff and Miss R. Thorner for analysis of the milk.

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Factors Affecting the Utilization of Food by Dairy Cows

6. The Rate of Contraction of the Reticulum

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(*Received* 29 *August* 1951)

In an earlier communication (Balch, Kelly & Heim, 1951) the influence of the reticulo-omasal orifice on the retention of food in the reticulo-rumen was considered. Data there presented showed that the closing of the orifice and the cycle of pressure changes in the omasum took place at the same rate as the contractions of the reticulum, irrespective of the activity of the cow. It is likely that the co-ordinated action of these three parts is largely responsible for the transfer of digesta from the reticulum to the omasum and for preventing backflow of digesta from the omasum to the reticulum. Phillipson (1939) reviewed the literature dealing with movements in the reticulo-rumen and then showed that although the contraction of the reticulum is usually part of a co-ordinated cycle of movements in all parts of the reticulo-rumen, variations occur in the part played by the rumen in this cycle, but only in some exceptional instances is the reticular contraction absent. Schalk & Amadon (1928) showed how these movements of the reticulo-rumen affected the arrangement and movement of digesta within it. The importance of the specific gravity of particles of food in determining the arrangement of digesta in the rumen was considered by Schalk & Amadon and by Balch & Kelly (1950). From these various investigations it is clear that the movement of digesta in and from the reticulo-rumen is determined by the movements of the walls of the two compartments and the nature of the food.

Schalk & Amadon (1928) stated that the rate of reticulo-ruminal activity was fastest during eating and slowest during rumination, with an intermediate rate when the animal was neither eating nor ruminating, merely 'resting'.

Earlier parts of the present investigation have shown that the efficiency of breakdown of food may be affected by the time the food remains in the reticulo-rumen (Balch, 1950) and the position it occupies (Balch & Johnson, 1950). An attempt was made, therefore, to find what variation occurred in rates of reticular contraction in cows receiving some of the diets used in our earlier investigations. The rate of contraction of the reticulum was determined during five types of activity: eating, standing ruminating, standing resting, lying ruminating and lying resting. It was thought that an investigation of this type would show whether variations in reticulo-ruminal motility, resulting from the different diets, were likely to change the arrangement of digesta within, or the rate of flow of digesta from, the reticulo-rumen. The rate of reticular contraction was used as an index of reticulo-ruminal motility.

METHODS

Cows and their treatment. Observations were made on two Dairy Shorthorn cows in which large rumen fistulas had been established; these cows had been used in previous experiments (Balch, 1950; Balch & Johnson, 1950; Balch & Kelly, 1950). The diets were similar to those used by these authors and also by Balch, Balch, Bartlett, Johnson & Rowland (1952). The concentrates were a mixture in the proportions of ground dredge corn (oats and barley) 100, linseed cake 50, weatings 25, undecorticated groundnut cake 25, decorticated groundnut meal 25, limestone flour 2 and common salt 1, parts. The thyroxine was given in four cubes mixed with the concentrate mixture, or eaten from the hand, two cubes twice daily. This dose supplied 100 mg L-thyroxine (see Balch *et al.* 1952).

Recording of reticular contractions. The activity of the reticulum was recorded by means of small toy balloons held in place by brass weights of about 750 g. From the balloons, pressure tubing passed through the fistulas to the tambours placed vertically in an Evershed and Vignole recorder. The tambours were fitted with pens which provided a continuous record, at 0·5 in. (12·7 mm)/min, of pressure changes in the reticulum for periods of up to 26 h. A more detailed description of this recorder has already been given by Balch & Kelly (1950).

The rumen fistulas were closed by means of the cannula and bung described by Balch & Johnson (1948); the bung used during the recording had a special opening through which the pressure tube passed.

Recording of jaw movements. While the cows were receiving diets in which all the hay was ground, and in a few instances while they were receiving other diets, the movements of the lower jaws were recorded. For this purpose the cows wore a leather head stall with a strip of lightly inflated bicycle inner tube fixed inside the muzzle strap. When the jaws opened the air pressure inside the tube increased, operating a small electrical switch clamped to the head stall and controlling the flow of current in a 4 V circuit which included a solenoid operating a pen in the recorder. Since these observations were made this system has been found less satisfactory than a tambour operated by a balloon on the muzzle-strap.

Examination of recorder charts. In summarizing the data collected, the paper charts from the recorder were examined in lengths representing 20 min of recording. The easily distinguished double contractions of the reticulum were counted as one, and the records were used to find how long the animal spent eating, resting or ruminating and whether she was standing or lying. During eating, the jaw movements were recorded as a long period of movement with few pauses, and there was a highly characteristic rapid rate of contraction of the reticulum. Rumination was shown as periods of regular jaw movement broken up by short pauses at regular intervals (Fig. 1) and by an extra contraction of the reticulum immediately preceding the normal double contraction and the start of each period of chewing. When the cows lay down there was a rise in the basal pressure in the reticulum, and the respiratory movements became more regular and prominent. Throughout the periods of recording, notes on the activity of the cows were added to the charts to provide a check on the interpretation of the records.

RESULTS

Details of the rate of contraction of the reticulum were collected during various types of activity while the cows were receiving three different diets. These diets will be considered separately.

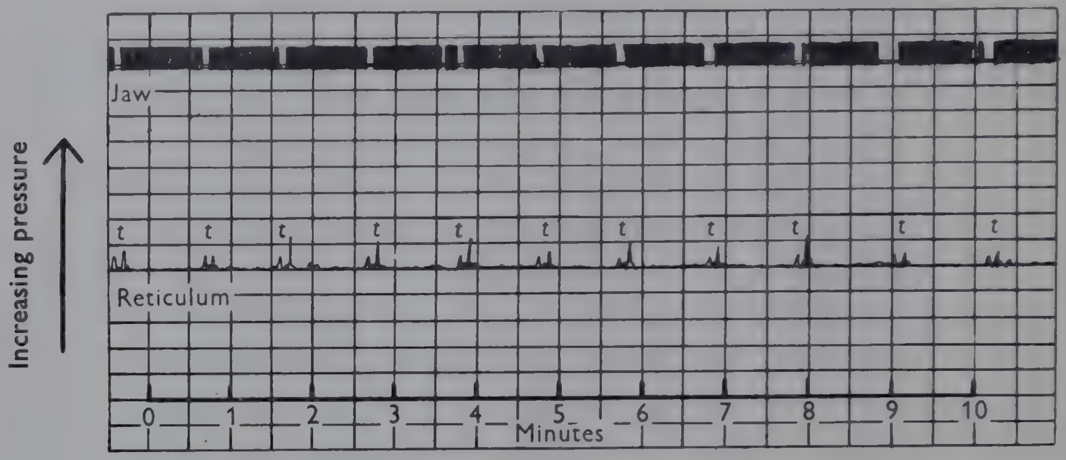

Fig. 1. Long-hay diet. Movements of the jaw, and pressure changes in the reticulum of cow Y standing ruminating. The triple contraction of the reticulum (*t*) is shown.

Table 1. *Numbers of reticular contractions and amounts of rumination in cows receiving long hay*

		Daily diet		Period of recording (min)	Total reticular contractions		Time spent ruminating (min)
Cow	No. of recording	Hay (kg)	Concentrates (kg)		Per period	Per h	
W	1	8·2	—	1380	1469	64	584
	2	8·2	—	1380	1528	67	785
	3	8·2	—	740	843	68	254
	Total		—	3500	3840	—	1623
	Daily mean		—	—	1580	66	668
Y	1	9·1	4·5	880	1019	70	383
	2	8·2	8·6	1400	1626	70	676
	3	8·2	8·6	840	932	67	334
	Total		—	3120	3577	—	1393
	Daily mean		—	—	1651	69	643

Long hay. Typical values for the total contractions of the reticulum of cows receiving normal diets, containing unground hay, are given in Table 1. The reticulum contracted at an average rate of 66/h in cow W and 69/h in cow Y. Both cows ruminated daily for about 11 h. The rate of contraction was fastest during eating (Table 2) and slowest during either resting or ruminating when the cows were lying down. When she was standing ruminating the mean rate of contraction in cow W was faster than when resting in either position. However, with this exception, possibly a false effect due to the small number of 20 min periods in which cow W was resting, the rate of contraction was slower during rumination than during resting.

On any one day the rates of contraction during different activities tended to be related; thus, if the rate of contraction was above the mean value for, say, eating it also was above the mean value for the other activities. The most constant rates of activity were observed during periods when the cows were lying ruminating. Variation in the rate of contraction while the cows were standing resting may have been partly due to the cows' eating during some periods small amounts of food remaining from the main meal. Since the jaw movements were not regularly recorded while the cows were receiving long hay, such spasmodic eating may have passed unobserved.

Table 2. *Mean rates of contraction of the reticulum, and heart rates of cows receiving long hay*

		Mean no. of reticular contractions/20 min* during					
			Resting		Ruminating		
Cow	No. of recording	Eating	Standing	Lying	Standing	Lying	Heart rate (beats/min)
W	1	26·0 (8)	21·0 (5)	18·4 (7)	24·4 (17)	18·3 (17)	Not recorded
	2	27·6 (15)	—	22·3 (7)	21·5 (4)	19·7 (27)	54
	Mean†	27·0	21·0	20·3	23·8	19·2	—
Y	1	30·4 (8)	27·0 (7)	23·7 (4)	19·5 (4)	18·7 (9)	Not recorded
	2	29·1 (16)	24·3 (9)	22·5 (4)	20·7 (15)	18·0 (23)	68
	Mean†	29·5	25·5	23·1	20·4	18·2	—

* Values in parentheses show the number of 20 min periods in which only the specified activity was recorded.

† Mean values have been weighted according to the number of 20 min periods used in arriving at the mean values for each period of recording.

Ground hay. When all the hay in the diet was finely ground the mean daily number of contractions of the reticulum was not markedly different from that observed with long hay. The average rate of contraction was 70/h in cow W and 68/h in cow Y (Table 3).

The ground-hay diet caused a marked decrease in the amount of true rumination. In cow W the triple contractions occurred, on the average, for only 63 min/day and in cow Y they were virtually absent. The typical pattern of reticular contractions and jaw movements found during the rumination of diets containing 16–20 lb. long hay (Fig. 1) was never normally observed while the cows were receiving the diets in which all the hay was ground. On the last day of recording with cow Y, true rumination was induced by placing a bristle brush in the reticulum (Fig. 2); the resulting periods of rumination occupied 28 min and involved twenty-eight triple contractions of the reticulum.

Although triple contractions of the reticulum were observed in cow W they were usually not accompanied by the periods of regular jaw movements found in true rumination. During the false rumination observed while this cow was receiving ground hay, the jaw movements were extremely irregular and bore little relationship

Table 3. *Numbers of reticular contractions and amounts of rumination and irregular jaw movements in cows receiving ground hay*

		Daily diet			Total reticular contractions		Rumination		Time spent in irregular jaw movements (min)
Cow	No. of recording	Ground hay (kg)	Concentrates (kg)	Period of recording (min)	Per period	Per h	Time (min)	Total triple contractions	
W	1	8·2	—	1480	1736	70	129	104	419
	2	8·2	—	940	1058	68	0	0	251
	3	8·2	—	940	1071	68	0	0	274
	4	8·2	—	640	764	71	45	49	178
	Total		—	4000	4629	—	174	153	1122
	Daily mean		—	—	1666	70	63	55	404
Y	1	9·1	4·5	1600	1792	67	0	0	90
	2	9·1	4·5	1320	1660	76	0	0	45
	3	9·1	4·5	1460	1618	67	0	0	36
	4	9·1	4·5	1800	1938	65	44*	38*	133
	Total		—	6180	7008	—	44	38	304
	Daily mean		—	—	1422	68	9	8	62

* In cow Y, rumination took place for 28 min including twenty-eight triple contractions, while a brush was in the reticulum (see p. 369).

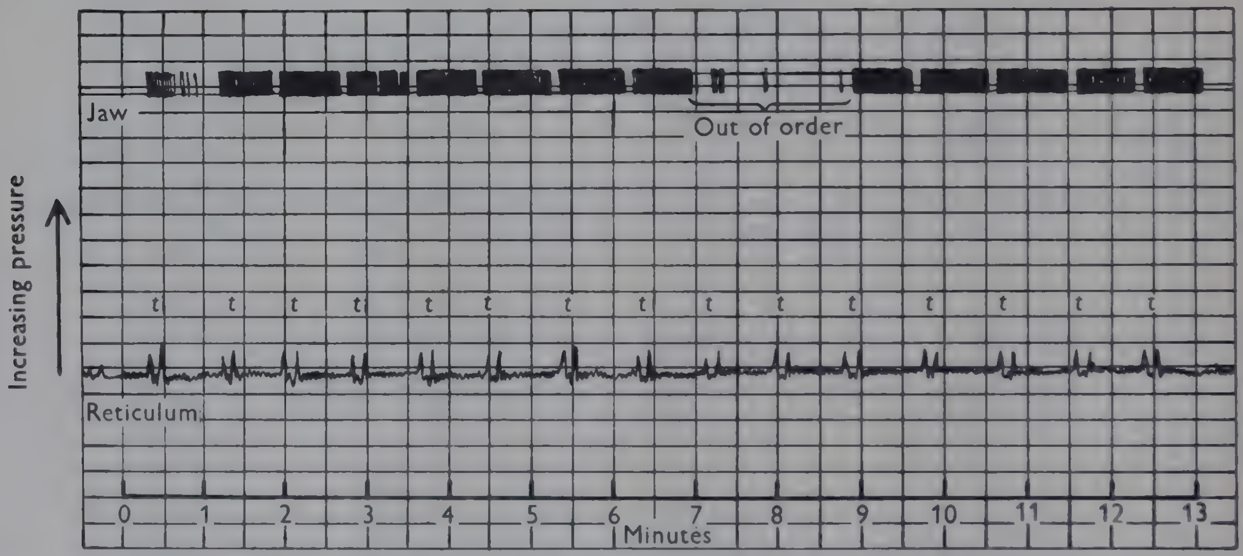

Fig. 2. Ground-hay diet. Movements of the jaw, and pressure changes in the reticulum of cow Y lying ruminating. This period of true rumination was induced by the presence of a brush in the reticulum. The triple contraction of the reticulum (*t*) is shown.

to the contractions of the reticulum (Fig. 3). Cow W devoted an average of 404 min/day and cow Y of 62 min/day to irregular jaw movements; in most instances these movements were accompanied by, but were not related to, the normal double contraction of the reticulum and by irregular belching (Fig. 4); this type of activity followed the insertion of a brush into the reticulum of cow W. Digesta appeared to be raised to the mouth by the irregular belching. Incomplete rumination, possibly of a similar type but lacking the contraction of the reticulum, was observed by Wester (1926) following atropin administration.

The rate of contraction of the reticulum during eating and resting was not markedly changed by ground hay (Table 4). The rate of contraction was again slower while the cows were lying resting than when they were standing resting, and during eating the rate of contraction was markedly faster than during resting; this was true not only for the mean values for each cow but also for seven of the eight separate periods of recording. The tendency for the rates of contraction during each type of activity to be all either fast or slow together on different days was again very noticeable.

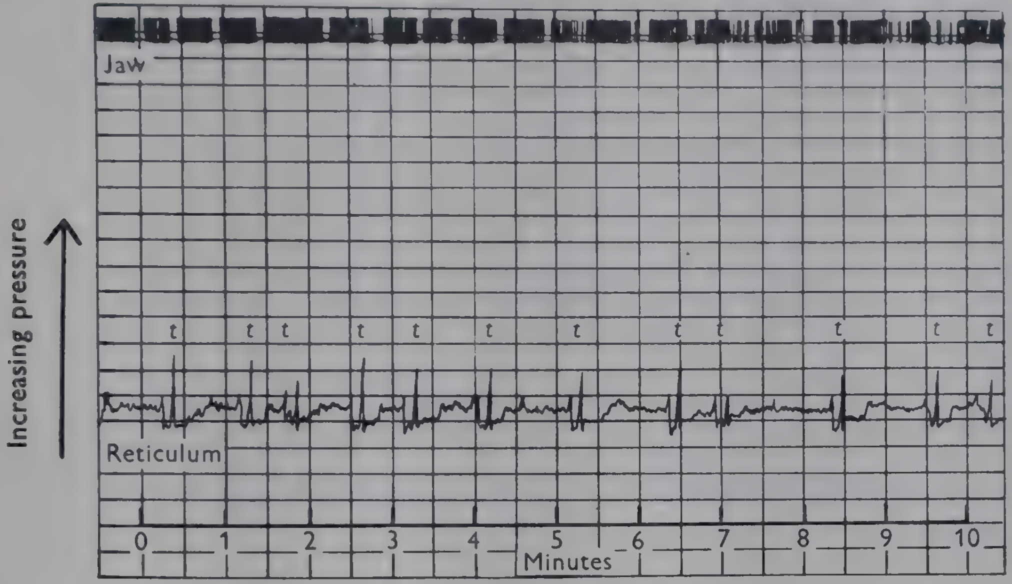

Fig. 3. Ground-hay diet. Movements of the jaw, and pressure changes in the reticulum of cow W standing attempting to ruminate. In this instance the reticulum had a type of triple contraction (*t*) but the periods of chewing were irregular.

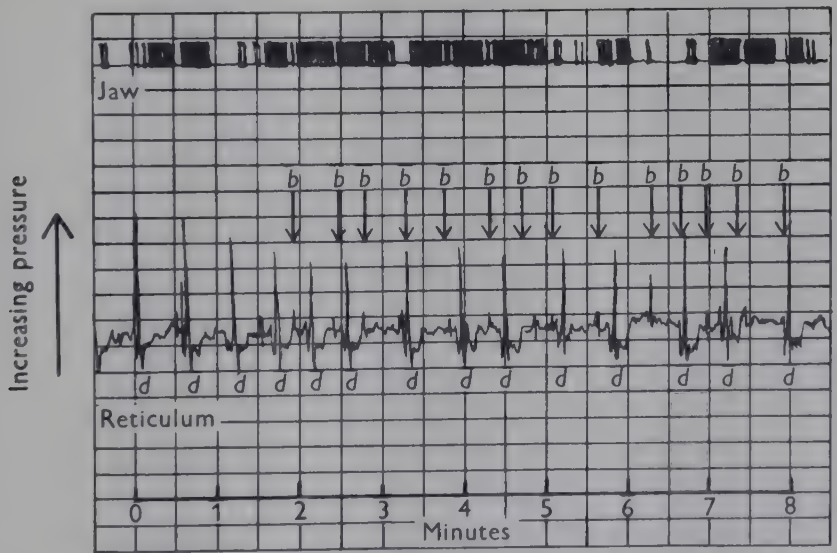

Fig. 4. Ground-hay diet. Movements of the jaw and pressure changes in the reticulum of cow W standing attempting to ruminate. This type of false rumination was characteristic of this cow with or without a brush in the reticulum (see p. 369). The double contraction of the reticulum (*d*) and the irregular belching (*b*) are shown.

Diets containing L-*thyroxine*. When the cows received daily 100 mg L-thyroxine there was a distinct increase in heart rate and, in cow Y, in milk yield. There was,

Table 4. *Mean rates of contraction of the reticulum of cows receiving ground hay*

		Mean no. of reticular contractions/20 min* during		
			Resting	
Cow	No. of recording	Eating	Standing	Lying
W	1	30·2 (8)	26·7 (9)	22·5 (39)
	2	25·1 (4)	20·2 (5)	21·4 (22)
	3	34·5 (2)	25·3 (9)	21·4 (23)
	4	28·2 (6)	23·7 (4)	21·3 (14)
	Mean†	29·0	24·6	21·8
Y	1	28·7 (8)	23·1 (16)	20·3 (36)
	2	32·6 (5)	26·2 (6)	23·1 (33)
	3	31·3 (6)	21·4 (14)	20·6 (30)
	4	27·2 (8)	21·9 (14)	20·1 (38)
	Mean†	29·6	22·7	21·0

* Values in parentheses show the number of 20 min periods in which only the specified activity was recorded.

† Mean values have been weighted according to the number of 20 min periods used in arriving at the values for each period of recording.

Table 5. *Numbers of reticular contractions and amounts of rumination in cows receiving* L-*thyroxine*

		Daily diet				Total reticular contractions		Rumination	
Cow	No. of recording	Hay (kg)	Concentrates (kg)	Thyroxine (mg)	Period of recording (min)	Per period	Per h	Time (min)	Total triple contractions
W	1	8·2	—	100	1280	1388	65	530	534
	2	8·2	—	100	1200	1344	67	438	435
	3	8·2	—	100	1580	1922	73	531	556
		Total	—	—	4060	4654	—	1499	1525
		Daily mean	—	—	—	1651	69	532	541
Y	1	7·3	6·8	100	1160	1442	74	470	396
	2	7·3	6·8	100	1480	1885	76	481	401
	3	7·3	6·8	100	1560	1797	69	630	501
		Total	—	—	4200	5124	—	1581	1298
		Daily mean	—	—	—	1757	73	542	445

therefore, no reasonable doubt that the metabolism of both cows was considerably raised by the thyroxine. Recordings of reticular motility (Table 5), compared with those in Table 1, showed that the mean number of contractions rose by 3/h in cow W and 4/h in cow Y during thyroxine feeding. However, the considerable variation between values obtained in the different periods of recording suggested that these differences were not significant. This difference in the rate of contraction was due, apparently, to less time being spent ruminating, the reduction being 136 and 101 min/day in cows W and Y, respectively (cf. Tables 1 and 5), and also to a tendency for the rate of contraction to be more rapid during eating and resting (cf. Tables 2 and 6). However, in cow Y there was also a tendency for the reduced amount of rumination to take place with a slower rate of reticular contraction.

Table 6. *Mean rates of contraction of the reticulum and heart rates of cows receiving* L*-thyroxine*

		Mean no. of reticular contractions/20 min* during					
			Resting		Ruminating		
Cow	No. of recording	Eating	Standing	Lying	Standing	Lying	Heart rate (beats/min)
W	1	26·1 (11)	25·2 (2)	20·5 (6)	20·0 (1)	20·4 (13)	66
	2	27·6 (14)	—	21·7 (10)	—	20·3 (11)	66
	3	31·9 (7)	27·1 (8)	24·4 (8)	21·7 (6)	20·3 (3)	61
	Mean†	28·0	26·7	22·3	21·5	20·3	64
Y	1	32·9 (16)	20·0 (2)	22·0 (2)	17·0 (6)	15·9 (12)	74
	2	34·4 (18)	31·0 (1)	24·8 (4)	19·0 (4)	15·3 (9)	75
	3	32·1 (7)	29·9 (9)	24·0 (6)	16·6 (7)	15·0 (11)	80
	Mean†	33·4	28·3	23·9	17·1	15·4	76

* Values in parentheses show the number of 20 min periods in which only the specified activity was recorded.

† Mean values have been weighted according to the number of 20 min periods used in arriving at the mean values for each period of recording.

DISCUSSION

It is known that the feeding of ground hay reduces the time spent in rumination (Kick, Gerlaugh, Schalk & Silver, 1937). Observations made in the course of the present study have shown that the reduction in time spent ruminating is due to the absence, as a result of the grinding of the hay, of the stimulus necessary to initiate the regurgitation reflex, and the pattern of behaviour associated with the reflex, in true rumination. The study confirms the finding of Schalk & Amadon (1928) that the regurgitation reflex is initiated by the presence in the reticulum and anterior rumen of material such as hay, that scratches the mucosa lining the walls of those regions, particularly that surrounding the cardia. As a result of the removal of this stimulus, by grinding the hay, triple contractions of the reticulum were extremely rare, and even when they occurred the pattern of rumination was rarely complete.

When all the hay in the diet was ground there was no marked change in the average rate of contraction of the reticulum over periods of 24 h. The rate of contraction may have increased slightly during the administration of thyroxine. Apart from these slight differences the rate of contraction of the reticulum showed a considerable variation depending on the activity of the cow; mean values during the five major types of activity are given in Table 7.

If digesta tended to flow from the reticulum to the omasum at the time of each double or treble contraction of the reticulum (Wester, 1926; Schalk & Amadon, 1928; Phillipson, 1939; Balch *et al.* 1951) there is reason for supposing that the flow of digesta would be proportional to the rate of reticular contraction. This hypothesis therefore, would lead to the conclusion that the flow of digesta to the omasum was greatest during meals and least during rumination. Schalk & Amadon claimed to have detected, by palpation, a rapid transfer of concentrates to the omasum during the course of a meal, but no comparable transfer has yet been detected in the fistulated cows at this Institute. However, as boluses composed of concentrates tend to disintegrate in the rumen more rapidly than boluses composed of hay (Schalk & Amadon, 1928) it is likely that newly ingested concentrates would arrive in the reticulum and the adjacent parts of the anterior rumen, and would be, therefore, in a favourable position for transfer to the omasum; such transfer would be likely to take place more rapidly than the transfer of hay eaten at about the same time.

Table 7. *Mean rates/h of contraction of the reticulum during various types of activity in two cows*

	Diet		
Type of activity	Long hay	Ground hay	Long hay and thyroxine
Eating	84·6	87·9	92·1
Standing resting	69·6	70·8	82·5
Lying resting	65·1	64·2	69·3
Standing ruminating	66·3	—	57·9
Lying ruminating	56·1	—	53·4

It was surprising to find that the rate of reticular contraction did not alter markedly during administration of thyroxine. The administration of thyroid-active materials is known to increase the rate of emptying of the stomach and intestinal motility in non-ruminants; this has been discussed previously (Balch *et al.* 1952). If the small increases in the rate of contraction of the reticulum, observed in the present experiments, indicated a slightly faster passage of digesta from the reticulo-rumen to the omasum, they would explain the slight increase in the index of the rate of passage of stained hay through the reticulo-rumen observed by Balch *et al.* (1952) in three out of four cows.

The frequency of the cycle of contractions in the reticulo-rumen, as indicated by the rate of contraction of the reticulum, was not markedly different for the three diets studied. It is unlikely that this factor alone could have caused any major change in the arrangement of digesta in the reticulo-rumen. The rate of contraction of the reticulum, unsupported by any index of the relative power of the contractions forming the whole cycle, gives little indication of the amount of mixing to which the reticulo-ruminal contents were subjected. Because of the absence of long particles from the digesta in the reticulo-rumen of cows getting only ground hay, these digesta could be very much more easily mixed (Balch, 1950), and since the rate of contraction varied little between diets containing either long or ground hay it is certain that the digesta were mixed more thoroughly when the hay was ground. This offers an explanation for the suggestion (Balch, 1950) that ground hay, from diets in which all the hay was ground, tended to be retained in the reticulo-rumen as long as, or longer than, unground hay.

SUMMARY

1. The rate of contraction of the reticulum was studied in two cows with rumen fistulas. Pressure changes in the reticulum were recorded continuously for periods of up to 30 h.

2. Grinding all the hay in the diet did not alter markedly the mean rate of contraction of the reticulum, but caused a great reduction in the amount of rumination.

This reduction was apparently due to the absence of the stimulus for the regurgitation reflex.

3. The rate of contraction of the reticulum was most rapid during eating and least rapid during rumination, with an intermediate rate during resting. When the cows were lying, the rate of contraction, during either resting or ruminating, was slower than when they were standing.

4. When the metabolism of the cows was raised by the addition to the daily diet of 100 mg L-thyroxine, there were only slight increases in the rate of contraction of the reticulum.

5. It is likely that the cycle of reticulo-ruminal contraction produced a more complete mixing of digesta in the reticulo-rumen when the cows were receiving a diet in which all the hay was ground than when the diet contained long hay.

I wish to thank Mr G. Heim and Mr V. W. Johnson for help during these experiments.

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Nutrition and Gastro-intestinal Helminths in Sheep on Hill Grazing: The Effect of a Dietary Supplement on Faecal Worm-egg Counts, Worm Burden, Body-weight and Wool Production

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(*Received* 21 *September* 1951)

There are many publications on the effect of nutrition on gastro-intestinal helminth infestations in sheep. In haemonchosis in lambs Whitlock, Callaway & Jeppeson (1943) in the United States and Ross & Gordon (1933) in Australia have shown that good diets result in lower worm-egg counts in faeces and less severe disease. Fraser & Robertson (1933) recorded the finding of larger numbers of *Haemonchus contortus* in poorly fed lambs than in well-fed animals grazing the same infested pasture, and Fraser, Thomson, Robertson & George (1938) showed that experimentally infected lambs had a greater number of mature parasites at slaughter if poorly fed than if given a good diet.

It has been shown that sheep experimentally infected with *Trichostrongylus* spp. give less wool (Carter, Franklin & Gordon, 1946) and show poorer weight gain and reduced utilization of protein, calcium and phosphorus than worm-free animals (Franklin, Gordon & Macgregor, 1946).

In this country Taylor has repeatedly drawn attention to the importance of nutrition in parasitic gastro-enteritis in sheep and has recorded the finding of larger numbers of trichostrongylids in lambs dosed with infective larvas and kept on a diet of straw or hay than in animals given a full ration of hay and concentrates (Taylor, 1943). He has also shown that heavily infected lambs on a good diet excrete in the faeces fewer worm eggs, whereas animals on a poor diet show a progressive rise in worm-egg counts (Taylor, 1943).

EXPERIMENTAL

General. Most of the work referred to deals with artificial infection or with heavy natural infections. The present investigation was designed to study the effect of natural infection in a flock of hill sheep kept under ordinary farming conditions and to see the effect on the general condition of the animals and on their faecal worm-egg counts and worm burdens of a dietary supplement given over a long period of time. One-half of the ewes and their lambs was given no supplement and served as control. There was no interference with normal flock management. Under such conditions, with the control animals on a fair diet of hill pasture and a dietary supplement of moderate size given to the others it was not expected that very large effects would be obtained, but it was hoped that examination of the whole flock over a period of many months might show a significant difference that would indicate whether dietary supplements are likely to be effective under ordinary farming conditions. An account of the seasonal changes in worm-egg counts of the ewes in 1946–7 has appeared elsewhere (Cushnie & White, 1948).

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Animals and their management. A flock of fifty Blackface ewes, about 18 months old, was purchased in October 1946 and was divided by paired weighings into two groups of mean weights 101·8 and 101·9 lb. The former group was given a daily supplement of 1 lb./ewe of a mixture of one part oats and two parts linseed-cake meal. The composition of this supplement, calculated from Woodman's (1947) tables, was about 88% dry matter, made up of protein 23, fat 8, soluble carbohydrate 43, fibre 9·5 and ash 4·5%. The starch equivalent, calculated from the same tables, was 69·1. From this figure the net energy value for fattening of the daily supplement may be put at 740 Cal.

From October 1946 until the end of the year the ewes grazed in rotation a number of grass leys around this Institute and were mated to Blackface rams. Early in January 1947 they were transferred to Brimmond Hill, about 2 miles south-west of the Institute and reaching 870 ft. above sea-level. The flock grazed some 270 acres of rough pasture, along with whin and heather. At night the flock was brought from the hill and enclosed in a paddock of 9 acres. In the morning it returned to the hill. Mating and lambing took place on fields close to the Institute.

The lambs born of ewes given supplement shared their dams' supplement until they were weaned at about 4 months, when they received their own ration which was gradually increased until each lamb had 1 lb. daily.

No cases of clinical helminthiasis were observed and no anthelmintic treatment was given to any of the animals.

The ewes were studied over a period of 2 years and observations were made on the 1947 crop of lambs until they were about 18 months old.

Helminthological methods. Worm-egg counts were made once a week on faeces of ewes and lambs. Samples were taken directly from the rectum by the same person and counts of all the eggs of the *Strongyloidea* spp. were made within a few hours by a modified McMaster technique (Gordon & Whitlock, 1939) and expressed as total no. of eggs/g faeces. Counts of *Strongyloides papillosus* were not included.

Total worm-counts were made on eleven ewes (six given supplement and five without) which were slaughtered in October 1948 and on the forty-three surviving lambs of the 1947 crop (twenty-three with supplement, twenty without), sent for slaughter in October 1948 at the age of about 18 months. The stomachs and intestines were tied off and brought back to the laboratory for worm counts. The abomasum and intestines were opened and the contents emptied into glass vessels. The mucous surfaces were washed with water and gently scraped and the material so obtained was added to the contents, which were then passed through a wire sieve (80 strands/in.) and washed with water until free from colouring matter. The material left in the sieve was transferred to a glass vessel, made up to 2 l. with water, and a sample of 100 ml. was taken while the suspension was being thoroughly stirred. This sample was treated with formalin to give a final concentration of 2% formaldehyde and stored until a worm count was made and the total count calculated. This method is unsuitable for large parasites because of the difficulty of accurate sampling: had time permitted, it would have been preferable to have counted the large worms directly in the material in the sieve.

Body-weight and fleece weight. All animals were weighed fortnightly. Fleece weights were recorded at shearing time: there were two records for the ewes (July 1947 and June 1948) and one for the lambs (June 1948).

RESULTS

Egg counts

Ewes. The weekly faecal worm-egg counts over a period of 2 years are shown in Fig. 1. In order to minimize the variation between individual animals the counts are expressed as the geometric mean of the worm eggs/g faeces/ewe at each weekly sampling. Group A consisted of twenty-three ewes given supplement and group B of twenty-four animals without supplement. The remaining three ewes died during the period of observation.

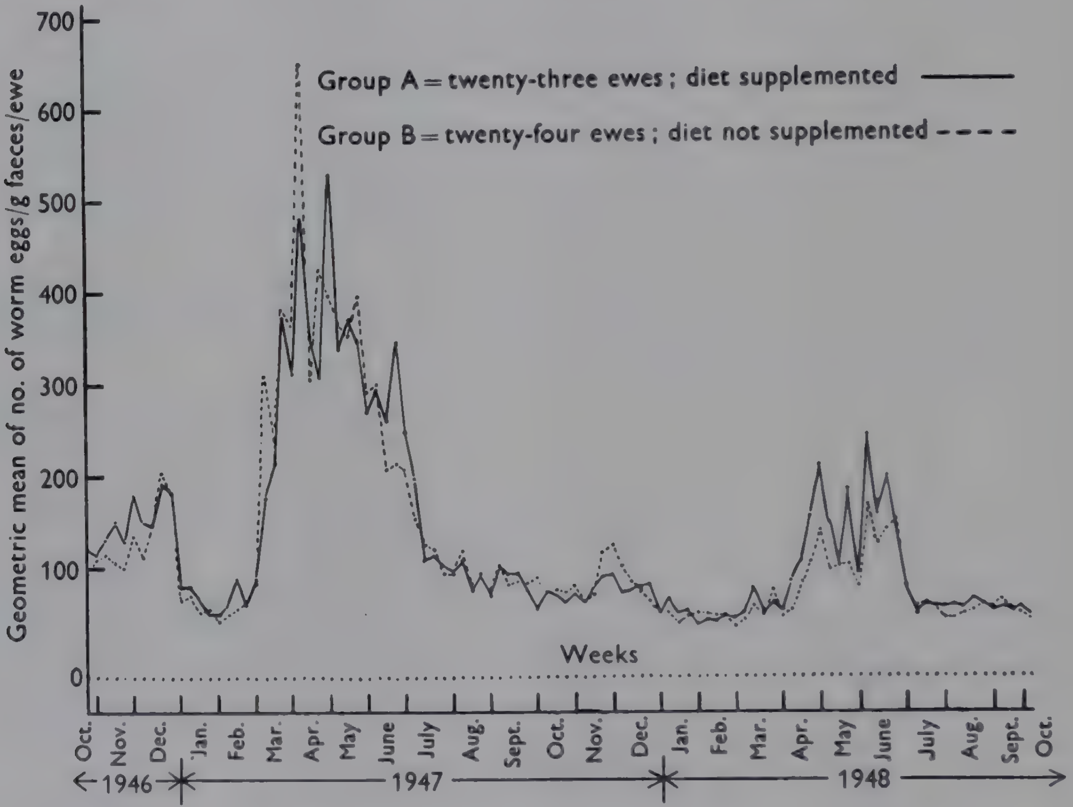

Fig. 1. Weekly egg counts of ewes from October 1946 to October 1948.

The mean initial counts for the supplemented and unsupplemented groups were 135 eggs per g (e./g) and 107 e./g, respectively. After a small rise in November and December 1946, the levels fell sharply on 30 December to less than 100 e./g. In early March a sharp rise occurred and reached its peak in the unsupplemented ewes on 1 April with a mean count of 647 e./g, the level in the other group being only 480 e./g. By the beginning of July 1947 the levels in both groups had fallen to just over 100 e./g and the spring rise had ended. From July 1947 until the end of March 1948 the levels in both groups varied only slightly. On 6 April 1948 a second spring rise began but it was much smaller than in 1947 and, as in that year, there was little difference between the two groups of ewes. By 29 June the levels had again fallen and remained around 70 e./g until observations ended in October 1948.

The general picture was of low resting levels and two spring rises, the first being much the larger. There was no striking difference between the supplemented and unsupplemented groups, either at their low resting levels or during the rises. Analysis of variance showed that the ratio of the geometric means of the two groups at no time reached significance and agreement between the two groups was remarkably close.

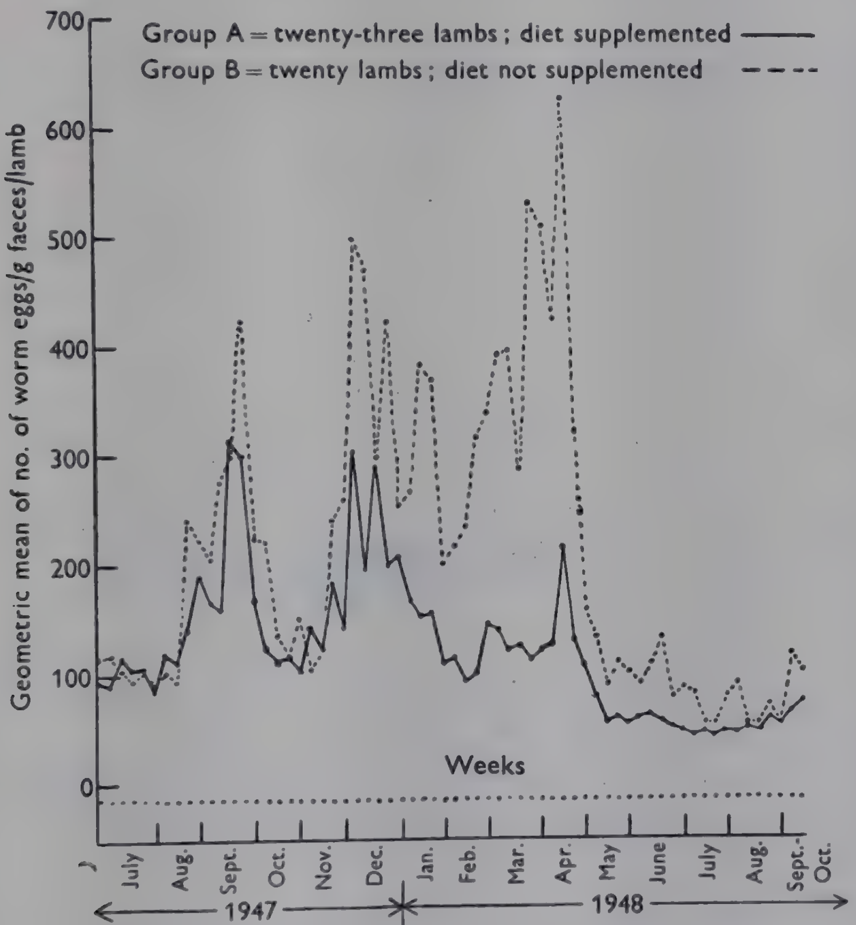

Fig. 2. Weekly egg counts of lambs born in 1947, from July 1947 to September 1948.

Lambs, 1947. The egg counts are shown in Fig. 2 and cover the period July 1947 to September 1948. The values are expressed as the geometric mean of weekly counts of worm eggs/g faeces/lamb. For the first 3 or 4 months after birth the egg counts were too low for the McMaster method to be used: only the counts obtained by this method have been recorded on the graph.

The initial levels were just over 100 e./g in both groups of lambs (twenty-three lambs given supplement and twenty without supplement). On 2 September 1947, a sharp rise occurred in both groups and reached its maximum on 30 September with a count of 430 e./g in the group without supplement, but with one only just exceeding 300 e./g in the supplemented group. Both groups had fallen to 100–150 e./g by the end of October. At the end of November the levels in both groups began to rise steeply. The unsupplemented group reached a maximum of 505 e./g on 9 December and did not fall to 100 e./g until 5 months later, by which time a large spring rise in March–April had taken place, with a maximum level of over 600 e./g. In contrast, the group receiving supplement recovered from the rise by January 1948, falling to a level of about 100 e./g and showed only a very small spring rise in April. The increasing peaks of the unsupplemented group were in striking contrast to the falling peaks of the group given supplement. By the end of May 1948 both groups had levels of 55–145 e./g and remained within these limits until the animals were sent for slaughter in October 1948.

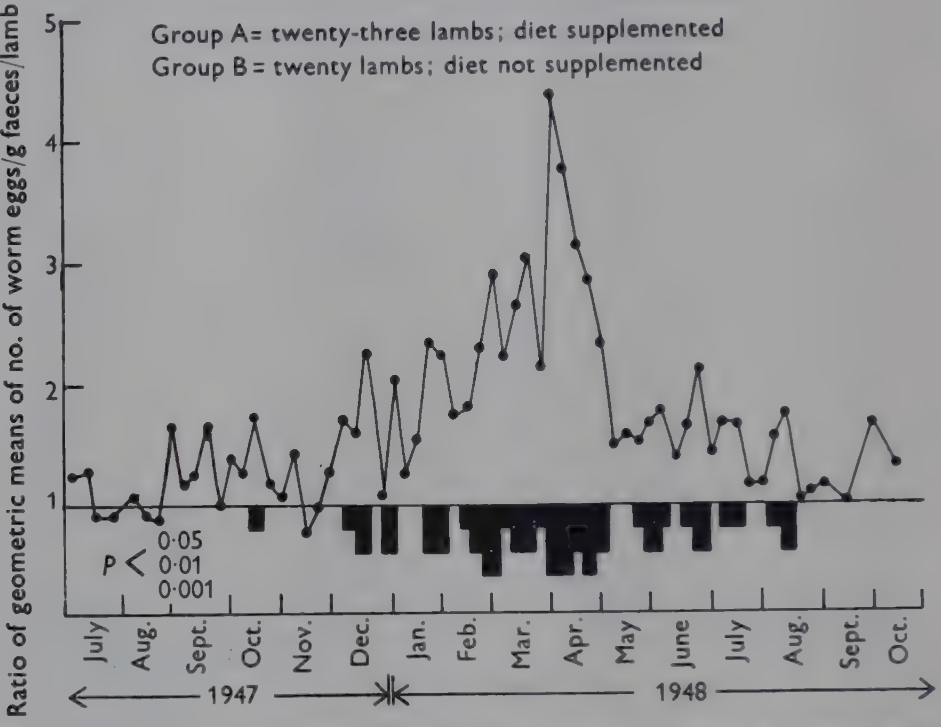

Fig. 3. Graph showing ratio of geometric means of weekly egg counts of the group of lambs without supplement to those of the group given supplement (horizontal line at 1 on ordinate). The values of *P*, indicating statistical significance, are given below the horizontal line.

Between 25 November 1947 and the end of the period of observation almost a year later the faecal worm-egg count of the supplemented group at no time reached that of the group without supplement. At the low resting levels the difference between the groups was small but during the three rises the differences were 105, 265 and 410 e./g, respectively.

This difference between the worm-egg counts in the two groups is shown in Fig. 3 in which the ratio of the geometric means of the egg counts is given. Nought in the McMaster count was for convenience replaced by 50 in order to facilitate the calculations, and this has reduced the significance at times when egg counts were low. Analysis of variance showed that the difference between the levels in the two groups was significant throughout most of the period between December 1947 and August 1948. During the spring rise of 1948 significance was at a high level ($P < 0{\cdot}001$) on several occasions and many weekly counts gave levels of $P < 0{\cdot}05$ or 0·01.

Total worm counts

Ewes. The total worm counts for six ewes given supplement and five without are shown in Table 1. The number of animals was too small to justify any conclusions, but it is of interest to observe the low worm burden. *Nematodirus* was not found in any animal and *Trichostrongylus* spp. were present in only four: in the lambs these two species accounted for most of the worm burden. The remaining thirty-six ewes were not slaughtered.

Table 1. *Total worm counts (calculated) in ewes*

Ewe no.	*Tricho-strongylus* spp.	*Ostertagia* spp.	*Cooperia* spp.	*Haemonchus contortus*	*Bunostomum* spp.	*Oesophagostomum* spp.	*Chabertia ovina*	*Nematodirus* spp.
				Ewes given dietary supplement				
249	0	0	40	0	0	0	0	0
251	640	100	200	0	40	0	0	0
228	110	940	0	0	80	0	0	0
220	0	20	20	0	0	0	0	0
224	120	360	0	0	20	0	0	0
255	0	20	0	0	0	0	0	0
Total	870	1440	260	0	140	0	0	0
				Ewes given no supplement				
260	0	0	0	0	0	0	0	0
211	0	120	0	0	60	0	0	0
246	100	220	20	0	100	0	0	0
259	0	0	0	0	40	0	0	0
225	0	220	40	0	20	0	0	0
Total	100	560	60	0	220	0	0	0

Lambs. Worm counts were low (Table 2). The relative numbers of the different species of helminths were similar in the two groups. *Trichostrongylus*, *Nematodirus* and *Ostertagia* were the most numerous species. *Haemonchus* was found in only one lamb. *Bunostomum* was a common parasite. More worms were found in the group of twenty lambs not given supplement than in the supplemented group of twenty-three lambs, but individual differences were marked and the difference between the two groups was not statistically significant.

Body-weight

There was no significant difference between the ewes in the two groups. At birth the mean weight of the lambs in the group from ewes given supplement was 6·63 lb. and of the lambs from control ewes 6·11 lb. Lambing took place over a period of about 1 month (March to April 1947) and because of the variation in ages of the lambs the statistical test of body-weight gains was restricted to the last three fortnightly weighings before slaughter in October 1947, by which time the slight variation in lambing dates could be ignored. Table 3 gives the mean of the last three weighings and shows that the difference in favour of the group receiving supplement is significant at a level of 0·1% ($P < 0{\cdot}001$), the mean difference in weight being 15·8 lb.

Table 2. *Total worm counts (calculated) in lambs*

Hogg no.	*Tricho-strongylus* spp.	*Ostertagia* spp.	*Cooperia* spp.	*Haemonchus contortus*	*Bunostomum* spp.	*Oesophagostomum* spp.	*Chabertia ovina*	*Nematodirus* spp.
				Hoggs given dietary supplement				
402	1080	160	0	0	40	40	40	1980
403	600	20	40	0	20	40	0	20
405	20	180	0	0	20	0	0	0
406	0	480	40	0	20	0	0	0
416	180	80	0	0	40	0	0	0
417	40	80	20	0	0	0	0	520
420	40	300	0	0	0	0	0	0
425	0	0	0	0	20	0	0	0
426	1140	60	0	0	0	0	0	0
429	360	40	20	0	40	0	0	20
432	320	440	40	0	0	0	0	0
433	80	20	80	0	40	0	0	0
435	20	60	20	0	0	0	0	0
436	0	320	60	0	0	0	0	0
442	160	420	0	0	20	0	0	0
450	120	20	40	0	0	20	0	0
451	300	60	80	0	0	0	0	0
453	240	0	40	0	40	0	0	0
454	480	800	20	0	0	20	0	20
459	120	540	0	0	60	0	0	20
465	280	0	40	0	0	0	0	0
469	120	20	0	0	0	40	0	0
470	780	40	0	0	20	0	0	0
Total	6480	4140	540	0	380	160	40	2580
				Hoggs given no supplement				
412	0	240	0	0	20	0	0	0
413	220	980	0	0	0	0	0	0
419	2580	100	0	0	20	0	0	4840
421	0	20	0	0	20	0	0	0
422	740	180	0	0	0	0	0	0
423	1600	420	0	0	40	0	0	1580
424	100	40	0	0	0	0	0	0
427	1760	2100	120	40	20	0	0	1240
430	60	200	0	0	40	0	0	0
437	220	60	40	0	40	0	0	20
439	680	60	0	0	40	0	0	0
441	40	40	80	0	0	0	0	0
445	40	0	0	0	20	0	0	0
446	1420	200	60	0	0	0	0	0
447	860	0	0	0	0	0	0	0
448	860	120	20	0	100	0	0	0
456	320	760	40	0	40	20	0	1080
457	80	20	80	0	40	0	0	0
464	240	360	0	0	20	0	0	4960
471	320	40	20	0	0	0	0	0
Total	12,140	5940	460	40	460	20	0	13,720

Fleece weight

Mean fleece weights for the ewes for shearings in 1947 and 1948, and for both years together, are shown in Table 4, also the weights for the 1948 shearing of the lambs born in 1947.

Both the ewes and the lambs showed a significant increase in fleece weight in the groups receiving supplement. The highest level of significance was in the lambs and in the 1947 fleece of the ewes ($P<0{\cdot}001$).

Table 3. *Mean body-weight of lambs*

	Unsupplemented	Supplemented	Difference with its standard error
Mean of last three fortnightly weighings (lb.)	97·4	113·2	15·8* ± 3·9

* Difference significant at 0·1 % ($P<0{\cdot}001$).

Table 4. *Mean fleece weights* (*lb.*) *of ewes and lambs*

Ewes	Unsupplemented	Supplemented	Difference with its standard error
1947	4·88	6·17	1·31* ± 0·296
1948	5·27	6·39	1·12† ± 0·385
1947 and 1948 (mean)	5·08	6·29	1·21* ± 0·241
Lambs			
1948	4·6	7·3	2·7* ± 0·4

* Difference significant at 0·1 % ($P<0{\cdot}001$). † Difference significant at 1 % ($P<0{\cdot}01$).

DISCUSSION

The experiment was carried out on a small flock of hill sheep kept under normal conditions of hill farming, using a breed well able to support itself and to produce lambs and wool under such conditions. No clinical symptoms of helminthiasis were observed; faecal worm-egg counts and worm counts were low and the control ewes and lambs maintained reasonable weight gains. Under such conditions a dietary supplement could not be expected to produce spectacular effects, and it is not surprising that no significant effect of the supplement could be demonstrated in the helminthological picture of the ewes which were already 18 months old when purchased.

The lambs given a daily dietary supplement showed striking differences in faecal worm-egg counts, body-weight and wool production, but not in total worm burden. The altered pattern of the weekly faecal worm-egg counts, with their higher levels and more pronounced peaks, in the control lambs suggests that the nutrition of lambs has a marked effect on egg production, especially during the spring rise and other seasonal rises.

The spring rise of the lambs given no supplement was about equal to that of the ewes in 1947, whereas that of the lambs receiving supplement was more like that of the ewes in the 2nd year of the experiment (1948). It is clear that the dietary supplement was associated with the reduced egg excretion of the lambs: the cause of the reduced spring rise in both groups of ewes in 1948 might be age resistance or specific immunity.

The cause of the spring rise is not known.* It might be caused by: (*a*) increased egg production of mature parasites already present; (*b*) development to maturity of parasites that have been lying dormant in the mucosa; (*c*) ingestion of fresh infective larvas. All three causes may be concerned. The third possibility (*c*) is unlikely because it necessitates the assumption that infective larvas are ingested in January to March when the temperature is most unfavourable to their survival outside the host. The lower resistance of animals at the end of the winter may well favour increased egg production of mature worms already present and also the development to maturity of immature forms that have lain inactive in the mucosa. Morgan, Parnell & Rayski (1950) suggest that excessive rain or snow favours high worm-egg counts in spring. If this is so it is not surprising that the high spring rise in the ewes should have occurred in 1947, for the winter of 1946–7 was a notoriously hard one, snow lay on the ground for some weeks and the temperature rarely rose above freezing-point (Cushnie & White, 1948).

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* *Note added in proof.* Since this paper was submitted for publication Morgan, Parnell & Rayski (1951, 1952) have found that the spring rise in egg counts in Scottish hill sheep is not due to larvas that have remained dormant in the abomasum and intestine throughout the winter but to the ingestion of infective larvas during March and April.

Fig. 2 shows that the difference in worm-egg counts between the group of lambs given supplement and the control group became progressively greater over the series of three seasonal rises in worm-egg excretion. The difference was greatest in March and April 1948, the period of the spring rise. Since the lambs had access to supplement from birth it is difficult to explain why its effect should appear to have been greatest some 10 months later when the lambs had already shown two seasonal rises in worm-egg counts. It would seem that the spring rise is the one most influenced by diet and is caused by a general rise in worm-egg production in spring due to the low level of resistance at the end of the winter. The other seasonal rises, in contrast, may be caused by particular species of helminths and be related to the ingestion of large doses of infective larvas from the pasture. In May and June 1948, when the temperature had become favourable to larval development and survival, the worm-egg counts of both groups of lambs fell steeply. It may be that warm weather and a good growth of grass have a beneficial effect on general health and resistance which is more than sufficient to offset the intake of larger doses of infective larvas. Recent work on the antibody response in natural worm infestations in sheep (Stewart, 1950) suggests that a fall in the egg count is accompanied by a rise in circulating antibody, an indication of the part which immunity may play in determining the level of the egg count.

It is likely that the three peaks in the worm-egg counts of the lambs in 1947–8 were caused by different species of parasite. Examination of larvas obtained from faecal cultures, and of species of adult worms obtained at autopsy at different times of the year support this view, although the helminthological picture of individual animals within a flock examined at any one time varies widely. Weekly worm-egg counts of a whole flock indicate general trends of egg counts, in spite of wide variations between individual animals. Not all the ewes and lambs showed the rises in worm-egg count shown on the graph of mean egg counts; some animals maintained a uniformly low count throughout the period of observation. It would seem that each animal has its own pattern of worm population and egg excretion, but that study of a sufficiently large number of animals in a flock will show a general trend over a period of weeks or months. Examination of total worm counts shows a variation within a flock that is even greater than with egg counts and is of significance only when large numbers of parasites are present.

Elimination of adult worms in the faeces was not investigated. Few worms were noticed among the hundreds of faecal samples examined. Tetley (1949) states that expulsion of adult worms is associated with rapid falls in egg counts and records the expulsion of some 45,000 worms from a lamb over a period of 30 weeks. Whether such a phenomenon follows the spring rise is worth investigation.

These observations show that the worm-egg count in lambs can be reduced and the spring rise almost prevented by the use of a simple daily dietary supplement. A weekly supplement might be adequate and would be more likely to be of practical value. The cost of the supplement was twice the value of the extra wool and mutton it produced. Supplement for half the year (winter only) would be an economic possibility and might do all that is needed. The reduction of worm-egg counts is of value in reducing contamination of pastures. It is of interest that in spite of the use of a 9-acre field every night by the flock for 2 years, with the probable establishment of a high level of infective larvas on the pasture, worm counts and egg counts remained low. Continual grazing kept the grass short and hardly a square foot of the field was free from sheep faeces.

The effect of the supplement on body-weight and fleece weight in the lambs was considerable and showed that the increased return in meat and wool that can follow an improved diet is worth consideration. It is probable that with heavier parasite levels the supplement could have a greater effect on the worm picture. Whether viewed in terms of increased production of meat and wool or as a contribution to reducing faecal worm-egg excretion and contamination of pastures, the use of a dietary supplement has much to recommend it.

SUMMARY

1. Half of a flock of fifty Blackface ewes on hill grazing, receiving a daily supplement of 1 lb. of a mixture of one part ground oats and two parts linseed-cake meal, gave wool yields significantly more than animals in the other half, receiving no supplement. No significant effect on body-weight, worm burden or weekly faecal worm-egg counts was observed.

2. Lambs of this flock of ewes, given access to the supplement from the time of birth and receiving increasing amounts up to 1 lb. daily after weaning, had reduced faecal worm-egg counts, smaller seasonal rises in worm-egg production and highly significant increases in body-weight and wool production as compared with lambs not receiving supplement.

3. Throughout the period of the experiment, which lasted for 2 years with the ewes and 18 months with the lambs, faecal worm-egg counts and total worm counts were low and no signs of clinical helminthiasis were observed.

The authors are indebted to Mr W. Thomson and his staff at this Institute for the husbandry of the animals and for the fleece and body-weight records and to Mr M. H. Quenouille of Marischal College, University of Aberdeen, for the statistical analysis.

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Observations on the Vitamin Metabolism of the Common Fowl

2. The Effects of Oestrogen and Progesterone Injections in Immature Pullets on the Riboflavin Content of the Magnum

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(*Received* 28 *September* 1951)

Riboflavin exists in the body of the fowl as the free vitamin, as flavin-mononucleotide and as flavin-adenine-dinucleotide. When a bird is about to ovulate, the amount of free riboflavin in the magnum rises to about 40% of the total vitamin present; after the egg has passed through the magnum and received its layers of albumen, the amount of free riboflavin in the magnum falls to about 30% of the total vitamin present. This decrease in free riboflavin content is approximately equal to the amount in the albumen of the eggs laid (Bolton, 1952).

The fluctuations in the quantity of free riboflavin in the magnum are most likely controlled by the endocrine system, probably by ovarian hormones. Fraps, Hooker & Forbes (1948) found progesterone in the blood plasma of laying hens and suggested that this hormone was of ovarian, and most likely follicular, origin. Hertz, Dhyse & Tullner (1949) showed that, while the biotin content of the blood serum of chicks was increased about fivefold by stilboestrol treatment, no avidin was formed in the oviduct. The simultaneous injection of stilboestrol and progesterone evoked both an increase of biotin in the blood serum and formation of avidin in the oviduct, thus suggesting that progesterone is involved in the secretion of egg albumen.

It is known (Common & Bolton, 1946; Common, Rutledge & Bolton, 1947–8; Common, Bolton & Rutledge, 1947–8) that injections of oestradiol dipropionate will increase the serum-riboflavin concentration of immature pullets about fifteenfold and (Bolton, 1950) that this increase is almost entirely due to an increase in free riboflavin.

It is also known (Bolton, 1951) that the higher level of riboflavin in the serums of laying birds, compared with non-laying birds, is due almost entirely to free riboflavin. Thus the changes in serum riboflavin content that occur as the fowl comes into lay can also be evoked by the administration of oestrogen.

The experiments described in the present paper were undertaken to ascertain the effects of oestradiol dipropionate and progesterone on the free riboflavin content of the magnum.

EXPERIMENTAL

Animals and diets. Brown Leghorn pullets were bred at this Centre and came from a single strain. All groups were reared under the same conditions. During the experimental period the birds were housed separately in all-metal laying cages and received the same daily ration of an all-mash laying diet, made up as follows: ground wheat 26, ground yellow maize 26, ground oats 25, wheatfeed 10, fish meal 4, earthnut-cake meal 6, cod-liver oil 1, precipitated chalk 1·75 and common salt 0·25 parts. Hydrated manganese sulphate (20 g) was added to each 100 lb. mash. This diet contained 1·7 μg riboflavin/g food and 1·0% calcium.

Pullets at this Centre are recorded as having laid when 18 weeks old (Greenwood & Blyth, 1946). The deposition of yolk and the hypertrophy of the oviduct would have begun when these birds were about 16 weeks old. Therefore, to ensure that the effects evoked by the hormone treatments are not affected by normal changes, birds should not be older than 16 weeks at the conclusion of the experiment and should preferably be slightly younger. For this reason the experiments were begun when the birds were 11 weeks old.

Hormones. The hormones used were oestradiol dipropionate and progesterone. The progesterone was dissolved in the same oily base (arachis oil) as that used for the oestradiol dipropionate and the oil administered was always brought to the same volume by adding a suitable amount of diluent. The need to do this has additional importance because of the observations of Crafts (1942) and Bruce & Tobin (1940) that injections of sesame oil caused a reduction of testis weight and so acted like an oestrogen. The choice of arachis oil as the solvent for the hormones was fortunate, for Russell (1952) has shown that injecting ethyl oleate into rabbits leads to the formation of 'cheesy' deposits at the site of injection. This finding has cast doubt on the efficacy of hormone absorption from this medium.

The hormone solutions were injected into the pectoralis muscles, the route used for injections of oestrogen and androgen by Common, Bolton & Rutledge (1947–8) and Common, Rutledge & Bolton (1947–8).

Analytical methods. The riboflavin content of the diet was computed from the amounts in the ingredients, which were assayed by the method of Kodicek & Wang (1949). Blood-serum riboflavin was determined by the method for total riboflavin of Burch, Bessey & Lowry (1948), and the partition of riboflavin into free vitamin, flavin-mononucleotide and flavin-adenine-dinucleotide in the magnum and liver by that of Bessey, Lowry & Love (1949). Macromodifications of the last two methods were adopted, as the microfluorimeter necessary for the original methods was not available. Serum-calcium determinations were made by the modified method of Halverson & Bergeim as described by Peters & Van Slyke (1932). It is necessary to remove protein from the serums of birds treated with oestrogens before attempting to precipitate calcium oxalate. Calcium in the food and faeces was estimated by Godden's (1937) method. The dry-matter content of the liver was ascertained by drying overnight at 100° and the fatty-acid content of this tissue by the method of McCance & Shipp (1933). Comb size is expressed as length plus height and the distance between the pelvic bones was directly measured during dissection.

Design of experiment. Groups of six birds were used in each of three experiments. The first bird of each group received oil alone and acted as a control, the second received a single dose of oestrogen, and the third a double dose. The remaining three birds all received progesterone, and of them one received no oestrogen, one a single and one a double dose. The birds were injected on alternate days until a total of six injections had been given to each: they were killed before the morning feed on the day following the last injection.

RESULTS

The results set out in Table 1 are the means of those obtained for birds in all three groups. They show that oestrogen administration evoked the usual hypertrophy of the oviduct and that the oviducts from the birds receiving the higher level of oestrogen alone were comparable in length and weight with those found by Common, Bolton & Rutledge (1947–8) and Common, Rutledge & Bolton (1947–8) after the same dose of oestradiol dipropionate. On the other hand, these workers found that the simultaneous injection of androgen had a synergistic effect on the growth of the oviduct, whereas in these experiments simultaneous injections of progesterone had an antagonistic effect. The riboflavin contents of the magnum, liver and blood serum are set out in Table 2.

DISCUSSION

Initial and final live weights. The results show that there was no significant difference between groups in either initial or final live weight, indicating that the hormone treatments had not had any effect.

Ovaries. Accurate dissection of the ovaries was not attempted, but the appearance and weights confirmed that they were quiescent in all birds. The other changes observed can therefore be taken as due to the experimental treatments.

Comb development. Progesterone alone did not have any effect on comb growth; oestrogen alone or in conjunction with progesterone caused a regression in comb size.

Spleen. The spleens from the birds receiving oestrogen, alone or in conjunction with progesterone, were paler in colour than those of the control birds or of birds receiving progesterone alone; the differences in weight were not significant.

Pelvic distances. The distance between the pelvic bones increased with administration of oestrogen, an increase that was not affected by progesterone treatment.

Liver. The amount of dry matter in the livers was not affected by either oestrogen or progesterone. The livers of the birds receiving oestrogen were heavier than those of

Table 1. *Effect of injections of oestradiol dipropionate and progesterone on pullets*

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Least significant difference ($P=0·05$)
Dosage of oestradiol dipropionate (mg)	6×0	6×1	6×2	6×0	6×1	6×2	—
Dosage of progesterone (mg)	6×0	6×0	6×0	6×1	6×1	6×1	—
Live weight:							
Initial (kg)	0·67	0·67	0·67	0·66	0·71	0·69	0·13
Final (kg)	0·75	0·75	0·74	0·73	0·75	0·75	0·14
Comb size:							
Initial (mm)	32	29	35	37	35	29	—
Change (mm)	+5	−1	−3	+6	−2	−1	2·7
Ovary:							
State	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	—
Weight (g)	0·20	0·17	0·15	0·20	0·14	0·17	0·04
Oviduct:							
State	Undeveloped	Developed magnum with white corded appearance	Developed magnum with white corded appearance	Undeveloped	Developed magnum with white corded appearance	Developing magnum with white corded appearance	—
Weight (g)	0·13	7·62	10·45	0·37	7·24	6·76	2·21
Length (mm)	65	275	360	93	250	232	39
Spleen weight (g)	1·19	1·13	0·93	1·28	1·04	0·82	0·70
Pelvic distance (mm)	11	15	28	11	17	31	7
Liver:							
Weight (g)	16·4	20·2	23·7	15·6	19·8	25·1	3·0
Dry-matter content (%)	29·3	30·4	30·5	29·0	30·3	30·8	3·2
Fatty-acids content (percentage of fresh tissue)	3·69	5·14	5·71	3·62	5·02	6·50	0·55
Serum calcium (mg/100 ml.)	12·6	45·3	73·1	13·0	40·3	69·7	24·8
Calcium retention (g)	1·66	1·82	1·87	2·03	1·77	2·23	0·94

Table 2. *Riboflavin contents of magnum, liver and blood serum*

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Least significant difference ($P=0·05$)
Dosage of oestradiol dipropionate (mg)	6 × 0	6 × 1	6 × 2	6 × 0	6 × 1	6 × 2	—
Dosage of progesterone (mg)	6 × 0	6 × 0	6 × 0	6 × 1	6 × 1	6 × 1	—
Magnum:							
Weight (g)	—	2·61	3·66	—	2·27	2·02	1·19
Length (mm)	—	132	153	—	112	101	26
Riboflavin content of magnum:							
Free riboflavin (μg/g)	—	0·97	0·94	—	1·60	1·29	0·36
Flavin-mononucleotide (μg/g)	—	0·28	0·38	—	0·24	0·33	0·38
Flavin-adenine-dinucleotide (μg/g)	—	2·50	2·45	—	2·59	1·74	1·19
Free riboflavin (percentage of total)	—	25·2	25·1	—	37·6	39·5	11·4
Riboflavin content of liver:							
Free riboflavin (percentage of total)	8·9	11·3	10·8	12·5	8·2	8·8	4·8
Total riboflavin (μg/g)	20·6	19·3	17·6	22·4	19·6	18·2	4·2
Total riboflavin in tissue (μg)	341	379	418	346	407	458	85
Serum riboflavin (μg/ml.)	0·05	0·28	1·12	0·06	0·71	1·29	0·39

either the controls or the birds receiving progesterone alone. The fatty-acid contents of the livers showed an increase with oestrogen treatment, but progesterone was without effect. Common, Bolton & Rutledge (1947–8) also found that the livers of birds dosed with oestrogen were heavier and had a higher content of fatty acids than those of control birds, and György & Rose (1949) that oestrogen administration increased the fat content of rat liver, but that progesterone was without effect.

Oviduct. The usual hypertrophy of the oviduct was observed after oestrogen treatment. Statistical analysis also showed a significant interaction between the two hormones, suggesting that they acted antagonistically on the growth of the oviduct. A similar examination of oviduct weights quoted by Common, Rutledge & Hale (1948) showed that androgen and oestrogen acted synergistically. An experiment is in progress to ascertain what happens when all three hormones are administered together.

Serum calcium and calcium retention. The usual rise in serum calcium was observed after oestrogen treatment, but progesterone was without effect. Calcium retention was not affected by either hormone or by their simultaneous administration.

Serum and liver riboflavin. Oestrogen evoked the usual increase in serum-riboflavin content. The increase in serum riboflavin after simultaneous administration of oestrogen and progesterone was significantly greater than after oestrogen alone when a single dose of oestrogen was given, but not after a double dose of oestrogen. These experiments therefore leave it undecided whether or not oestrogen and progesterone act synergistically on the concentration of serum riboflavin.

The total riboflavin content of the liver was unchanged with increasing oestrogen dosage. It will be noted that the percentage of free vitamin in the liver did not show any appreciable change, thereby supporting the suggestion put forward (Bolton, 1952) that this organ does not play any part in meeting the demand for free riboflavin as the egg passes down the magnum.

The total content of riboflavin in the liver showed an increase with oestrogen dosage. It is therefore considered highly unlikely that the extra riboflavin in the blood serum and in the magnum could be due to the mobilization of body reserves, for it is most probable that the liver store would then be depleted rather than augmented. It is therefore concluded that the increased content of riboflavin in liver, blood serum and oviduct is a symptom of increased absorption or retention from the alimentary tract. This view is in accord with that of Common, Rutledge & Bolton (1947–8).

Magnum riboflavin. The degree of hypertrophy of the magnum was greater when oestrogen was administered alone rather than in conjunction with progesterone. The interaction between oestrogen and progesterone almost reached the 5% level of significance, suggesting that the antagonistic effects of progesterone on oestrogen seen in the whole oviduct may also apply to the magnum.

The hypertrophy of the magnum in birds receiving oestrogen was sufficiently great to permit the riboflavin present to be partitioned into free vitamin, flavin-mononucleotide and flavin-adenine-dinucleotide. The results show that the contents of flavin-mononucleotide and flavin-adenine-dinucleotide were of the same order, but that the content of free riboflavin was increased by the simultaneous administration of progesterone and oestrogen to a level above that produced by oestrogen alone.

To compare the results of this experiment with those obtained previously, the content of free riboflavin in the magnum was expressed as a percentage of the total vitamin present. When the birds were dosed with oestrogen alone, about 25% of the riboflavin was present as the free vitamin. The simultaneous administration of progesterone increased the percentage to about 40. These figures are in close agreement with the values of 40% of free riboflavin in the magnum just before ovulation and 30% after the egg has reached the shell gland (Bolton, 1952). As already mentioned, progesterone has been detected in the blood plasma of laying birds (Fraps *et al.* 1948).

The hypothesis is therefore put forward that the increased content of free riboflavin in the magnum occurring just before ovulation results from the actions of oestrogen and progesterone.

SUMMARY

1. The effects of oestrogen and progesterone injections in immature female fowls were studied.

2. Oestrogen alone evoked hypertrophy of the oviduct, a decrease in comb size, increases in liver weight and total liver riboflavin, increases in serum calcium and serum riboflavin and an increase in liver fatty-acid content.

3. Progesterone antagonized the oestrogen effects on the growth of the oviduct, and increased the percentage of free riboflavin in the hypertrophied magnum from 25 to 40.

4. It is suggested that the increase in the percentage of free riboflavin in the magnum of a bird about to ovulate is due to the simultaneous action of oestrogen and progesterone.

The author thanks Messrs Ciba Ltd., The Laboratories, Horsham, for presenting the arachis oil used in this study.

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Variations in the Composition of Egg-yolk Pigment

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(*Received* 10 *November* 1951)

Gillam & Heilbron (1935) in a study of the effects of feeding maize and grass on the composition of yolk pigment have shown that xanthophyll is the principal pigment of egg yolk, although cryptoxanthin and β-carotene are also present, the former sometimes in amounts that contribute appreciably to the total vitamin A activity of the yolk. In view of this, the extent of the variations in the cryptoxanthin and β-carotene of the yolk is of considerable importance, particularly the variations that may be experienced under the conditions normally adopted for poultry breeding. In the management of breeding stock the hens are almost invariably allowed free range over good pasture, the grass supplying a considerable, and often the greater, part of the hens' requirements for vitamin A.

In a previous paper (Grimbleby & Black, 1950) an experiment was described in which the variation in pigment and vitamin A contents of individual eggs was examined, the eggs being obtained from four birds taken from a breeding flock of Rhode Island Red hens fed on a pigment-free diet but allowed free range over good pasture. No vitamin A supplement was given. The yolk pigments from individual eggs were fractionated, xanthophyll, cryptoxanthin and β-carotene being separately determined. According to Kuhn, Winterstein & Lederer (1931) the so-called yolk 'xanthophyll' is a mixture of lutein and zeaxanthin, but the separation of the xanthophyll fraction into these components was not attempted. The vitamin A content of the yolk was also determined and the significance of the variations in total pigment and vitamin A contents of egg yolk was discussed. The present paper deals with the variations in composition of the yolk pigment found in the same experiment.

EXPERIMENTAL

The experimental procedure adopted for the extraction and separation of the yolk pigments has already been described (Grimbleby & Black, 1950), but it should be further stated that the identification of the pigments was established by mixed chromatograms, pure zeaxanthin and cryptoxanthin required for this purpose being obtained from *Physalis alkakengi* by the method of Kuhn & Grundmann (1933) and the β-carotene from carrots by the method of Strain (1934). As the separation of lutein and zeaxanthin was not effected by adsorption on magnesium hydroxide under our conditions, pure zeaxanthin was used to identify the position of the so-called xanthophyll fraction.

RESULTS

The results are shown diagrammatically in Fig. 1 and summarized in Table 1.

They show that the dihydroxycarotenes having no vitamin A activity, lutein and zeaxanthin, usually referred to as 'xanthophyll', are present in considerable amounts compared with the active pigments cryptoxanthin and β-carotene. The quantities found were in close agreement with those found by Gillam & Heilbron (1935) in bulk samples of yolk. The results show further that, although cryptoxanthin was almost invariably a constituent of yolk pigment, β-carotene was frequently absent; in the first eggs laid in spring it was, in fact, unusual for β-carotene to be present. The pigments were found in the eggs of the four birds in the average proportions shown in Table 2. The cryptoxanthin content of the eggs followed the xanthophyll content very closely, there being a highly significant correlation between the two (Table 3). Hen no. 162 stopped laying after only five eggs had been laid in early March and ceased production for 26 days before again coming into lay, with the result that the pigments in the eggs from this bird did not reach the low fluctuating level reached in those of the other three birds, which had maintained a continuous production of eggs over the whole experimental period.

Table 1. *Xanthophyll, cryptoxanthin and β-carotene contents of individual eggs from four Rhode Island Red hens*

Hen no.	Xanthophyll (mg/egg)			Cryptoxanthin (mg/egg)			β-carotene (mg/egg)			Av. wt. of yolk (g)	No. of determinations
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.		
43	1·17	0·36	0·68	0·042	None	0·019	0·006	None	0·002	19·6	42
46	1·60	0·31	0·74	0·047	0·010	0·023	0·007	None	0·004	19·2	38
107	1·18	0·33	0·52	0·044	0·008	0·015	0·005	None	0·002	18·4	39
162	2·36	0·29	1·27	0·071	0·013	0·047	Trace	None	Trace	20·7	21

Table 2. *Ratio xanthophyll: cryptoxanthin: β-carotene in yolk pigment*

(Average values)

Hen no.	Xanthophyll	Cryptoxanthin	β-carotene
43	1000	28	3
46	1000	31	5
107	1000	29	4
162	1000	37	Trace

Table 3. *Relation between the contents of xanthophyll and cryptoxanthin of individual eggs*

Hen no.	No. of determinations	Correlation coefficient	Significance
43	43	0·908	Highly significant
46	38	0·946	Highly significant
107	39	0·845	Highly significant
162	23	0·880	Highly significant

DISCUSSION

These results raise certain questions about the utilization of β-carotene and of cryptoxanthin by the hen and about the source of the yolk cryptoxanthin.

Kline, Schultze & Hart (1932), Record, Bethke & Wilder (1937) and others have shown that carotene and vitamin A are utilized with equal efficiency by the fowl, but Frey & Wilgus (1949) reported that, although vitamin A was superior to

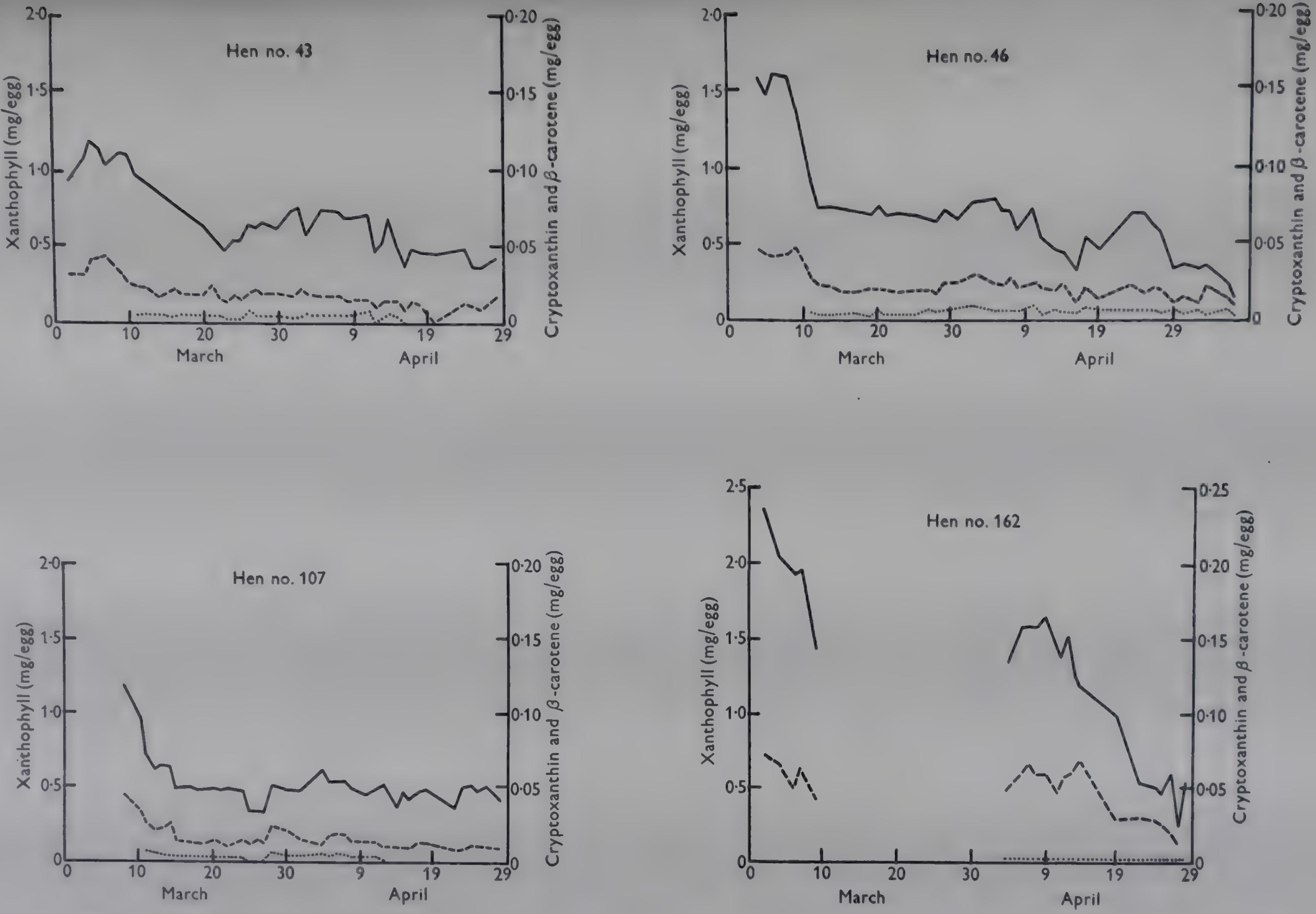

Fig. 1. Xanthophyll, cryptoxanthin and β-carotene contents of eggs from hens receiving a pigment-free diet supplemented by grass feeding. ——, xanthophyll; - - -, cryptoxanthin; · · ·, β-carotene.

carotene in building liver reserves of vitamin A, carotene from alfalfa was superior to vitamin A for the transfer of vitamin A and carotene to the egg. In the present experiment the vitamin A requirements of the birds were met entirely by the carotenoid pigments derived from grass, and a highly significant correlation between the total pigment and vitamin A content of individual eggs was, in fact, found (Grimbleby & Black, 1950). It must be assumed, therefore, that β-carotene was assimilated in amounts comparable with those of the inactive pigments, and the very small quantities of β-carotene in the eggs indicate that little of the β-carotene absorbed escaped conversion to vitamin A.

The absence of β-carotene from the first few eggs laid after a rest from egg-laying suggests that the pigments transferred to these eggs had been held in store by the hens for a sufficient length of time to permit the conversion of all assimilated β-carotene to vitamin A. When this store was somewhat depleted and pigments were transferred more directly from feed to egg, there was a tendency for some β-carotene to escape this conversion, but the amount was always small.

The quantities of cryptoxanthin found were sufficient to contribute appreciably to the total vitamin A activity of the yolk. The eggs from all birds contained on the average 0·023 mg cryptoxanthin, equivalent to 19 i.u. vitamin A (assuming that 0·0012 mg cryptoxanthin ≡ 1 i.u. vitamin A). The average vitamin A content of the same eggs was 182 i.u. so that the cryptoxanthin present, if utilizable by the fowl, would increase the total vitamin A activity of each egg by about 10%. The presence of such quantities of cryptoxanthin in the eggs, compared with the very small amounts of β-carotene, suggests that cryptoxanthin was not converted to vitamin A as readily as β-carotene, but the quantities of the various pigments ingested by the hens were not determined, so that no definite conclusion can be reached on this matter.

One point of considerable interest arising from these results concerns the source of the yolk cryptoxanthin. This pigment was present in all but one of the eggs examined; further, a highly significant correlation was found between the contents of cryptoxanthin and xanthophyll of individual eggs. The possibility that the presence of the cryptoxanthin was due to storage by the hens during a previous period of intake is discounted by the close correlation found between the cryptoxanthin and xanthophyll contents, as some, at least, of the latter must have been obtained from grass recently eaten. Further, Titus, Fritz & Kauffman (1938) have shown that the amount of stored pigment transferred to eggs falls progressively to insignificant amounts after about eleven eggs have been laid, whereas we found fluctuating amounts in eggs up to the fortieth egg laid. It would appear therefore that the two pigments were derived from the same source, yet, so far as we are aware, cryptoxanthin has not been reported to be a constituent of grass, and our examination of grass has not revealed its presence. It would appear therefore that the hen is able to alter other carotenoid pigments to cryptoxanthin, although from the data available it is not possible to say which carotenoid pigment is involved. The conversion of zeaxanthin or lutein to cryptoxanthin would mean that the hen does, in fact, utilize vitamin A-inactive pigments for the synthesis of vitamin A-active ones, as has been suggested by Virgin & Klussmann (1932).

SUMMARY

1. Successive eggs from four birds in a breeding flock of Rhode Island Red hens, maintained on a grain ration free from pigment and vitamin A but allowed free range over good pasture, were analysed for their xanthophyll, cryptoxanthin and β-carotene contents.

2. The pigment in egg yolk was mainly a mixture of lutein and zeaxanthin, usually referred to as xanthophyll. Cryptoxanthin and β-carotene were also present in small amounts.

3. A significant correlation was found between the cryptoxanthin and xanthophyll contents of eggs from hens receiving grass in the diet.

4. The significance of the presence of cryptoxanthin and the variation in the contents of the various pigments are discussed.

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Antibiotics and Liver Extract for Suckling Pigs

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(Received 15 November 1951)

In experiments previously described (Braude, 1949) we observed a growth-stimulating effect in suckling pigs dosed with a liver extract, whereas we obtained no response to dosing with pure crystalline vitamin B_{12} (Braude & Mitchell, unpublished results). Following reports from the United States of America about the growth-promoting effect of various antibiotics (Stokstad, Jukes, Pierce, Page & Franklin, 1949), we set out to investigate further the possibility of increasing the rate of growth of suckling pigs by supplementing their diet with antibiotics alone or combined with liver extract.

EXPERIMENTAL

General. The experiment was run on very much the same lines as the earlier ones. The test was carried out simultaneously on pigs reared either indoors or outdoors. Liver extract similar to that used in our previous trials was kindly put at our disposal by Glaxo Laboratories Ltd. The antibiotics used were penicillin and streptomycin, which were at the time the two most likely to be readily available in this country and were found in tests on chicks (Coates, Harrison, Kon, Mann & Rose, 1951) to be at least as effective growth stimulants as other antibiotics recently used in the United States.

Pigs and their treatment. Every suitable litter of pigs born in our piggery during the period May–November 1950 was included in this experiment; all were from home-bred Large White sows. Alternate litters were reared indoors, while the others were taken out when 3–4 days old to portable pig houses with runs, moved daily on pasture. The experimental unit within each litter consisted of six pigs chosen according to weight to form the most uniform group possible when the experimental treatment began at 14 days old. Usually one unit of six pigs per litter was available, but from a few large litters two units were used, the six heaviest pigs forming one unit and the six lightest the other. Except for the experimental dosing and weekly weighing, the litters were reared in the normal way. The litters reared indoors were dosed with iron pyrophosphate for 6 days beginning on the 4th day of life, and all litters were given unrestricted in a creep a suckling-pig meal mixture from the 3rd week onward. The meal mixtures fed to the sows and to the suckling pigs both contained 10% white fish meal. The following six treatments were allocated at random to the six pigs forming an experimental unit:

(1) Control.

(2) Daily dosing with 2 ml. liver extract, supplying 24 μg vitamin B_{12}.

(3) Daily dosing with a gelatin capsule containing 10 mg penicillin, given as 18 mg procaine penicillin.

(4) Daily dosing with a gelatin capsule containing 10 mg streptomycin, given as 15 mg of the calcium-chloride complex.

(5) Treatments 2 and 3 combined (liver extract and penicillin).

(6) Treatments 2 and 4 combined (liver extract and streptomycin).

Dosing. The experimental dosing was carried out once daily for 21 days, beginning on the 14th day of life. Altogether twenty-four units of six pigs were used, of which twelve were kept indoors and twelve out of doors. The pigs were weighed once weekly always at the same time of day, throughout their suckling period of 8 weeks.

RESULTS

Out of the 144 pigs used in this experiment six did not complete the test for reasons not connected with the experiment. Calculated values were inserted by the 'missing plot' technique (Yates, 1933).

Table 1. *Mean live-weight gains and responses to liver extract during 8 weeks suckling of pigs reared outdoors and indoors*

(Means adjusted for differences in weight at birth)

Antibiotic	Outdoor litters	Indoor litters	Mean
	Live-weight gain (lb.)*		
None	32·6	27·8	30·2
Penicillin	31·6	27·7	29·6
Streptomycin	32·7	27·9	30·3
S.E.	±0·93†	±0·76‡	±0·53
Mean	32·3	27·8	30·0
S.E.	±0·70		
	Response to liver extract (lb.)		
None	1·1	4·0	2·6
Penicillin	0·7	1·0	0·9
Streptomycin	−0·2	3·9	1·9
S.E.	±1·52		±1·07
Mean	0·5	2·9	1·7
S.E.	±0·87		±0·61

* Combined results for pigs with and without liver-extract dosing.

† Standard error for use in horizontal comparisons only.

‡ Standard error for use in all other comparisons.

Table 1 gives the mean live-weight gains and responses to liver extract, with their standard errors, of all pigs on experiment. The figures are adjusted for differences in weight at birth by means of a standard covariance analysis, the regression coefficient within litters of live-weight gain on initial weight being +4·28 ± 1·20.

Table 2 gives the statistical analysis of the results; they show that the rate of growth of pigs, reared either indoors or outdoors, was not affected by dosing with antibiotics. Dosing with liver extract had no effect on pigs reared out of doors, but pigs reared indoors grew at a significantly higher rate than their litter-mates not given liver extract. Pigs reared out of doors grew significantly faster than pigs reared indoors, independently of any other treatment given.

Table 2. *Mean squares in the analysis of variance* (*in terms of individual piglets*)

(Live-weight gain adjusted by covariance on initial weight)

	Degrees of freedom	Mean squares
Between litters:		
Blocks	11	82·67
Environment (*E*)	1	723·71*
Error	10	34·77
Within litters:		
Antibiotics (*A*)	2	5·63
$A \times E$	2	3·34
Liver extract (*L*) (indoors)	1	156·42*
Liver extract (*L*) (outdoors)	1	4·48
$A \times L$	2	8·44
$A \times L \times E$	2	11·12
Error	103	13·60

* $P < 0{\cdot}002$.

DISCUSSION

It is important to keep in mind that both the nursing sows and the suckling pigs used in this experiment received a balanced diet containing protein of animal origin. One should also remember that dosing with liver extract and antibiotics was limited to 21 days (the 2nd–5th weeks of the pig's life). Under these conditions it appears that no growth-promoting effect is obtained by supplementing the diet with either penicillin or streptomycin in the amounts used in this experiment. Our previous findings (Braude, 1949) that pigs reared indoors benefit from supplementing their diet with a small amount of liver extract, but that there is no similar effect on pigs reared out of doors, were confirmed. Similarly, the well-known and commercially important fact that pigs reared out of doors grow better than those reared indoors was again confirmed in this experiment.

We wish to thank Miss Z. D. Hosking for carrying out the statistical analysis of the data and Glaxo Laboratories Ltd for the liver extract and the antibiotics.

SUMMARY

1. Suckling pigs given daily during the 2nd–5th weeks of life 10 mg of either penicillin or streptomycin did not grow better during the 8-week suckling period than untreated litter-mates. The diets of mothers and young contained white fish meal.

2. Previous findings that pigs reared indoors benefit from the addition of a liver extract to their diet were confirmed.

3. Irrespective of treatment, pigs reared outdoors on pasture grew better than those reared indoors.

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PROCEEDINGS OF THE NUTRITION SOCIETY

SEVENTY-SECOND SCIENTIFIC MEETING—THIRTY-THIRD SCOTTISH MEETING

DEPARTMENT OF PHYSIOLOGY, UNIVERSITY COLLEGE DUNDEE, UNIVERSITY OF ST ANDREWS

2 FEBRUARY 1952

BONE AS A SKELETAL STRUCTURE AND AS A MINERAL RESERVE

Chairman: PROFESSOR R. B. HUNTER, *Department of Pharmacology and Therapeutics, Medical School, Dundee, University of St Andrews*

The Evolution of Bone as Tissue and Organ

By P. A. ORKIN, *Natural History Department, University of Aberdeen*

If a symposium such as this had been held 50 years ago, it is certain that a contribution on the evolution of bone would have differed greatly from the present one. In those days ideas about the origin of bone and the early history of the vertebrates had reached a condition of relative stability in which available facts and current theory were harmoniously blended. It was believed that the vertebrates were descended from some lowly type like *Amphioxus*, with no differentiated head or jaws, and with a skeleton composed of a few simple, cartilaginous rods. From these were descended the jawless cyclostomes, creatures rather like the modern lampreys, and from these, shark-like fishes with jaws and well-developed skeletons of cartilage. The next step in the rise of the vertebrates was thought to be due to an evolutionary novelty, the appearance of bone. This made possible very strong and well-articulated skeletons capable of withstanding the stresses of an active life in the water, or of bearing the weight of a heavy body on land.

On the whole the fossil evidence was in agreement with this evolutionary picture. Then too, theoretical zoology was dominated by Haeckel's biogenetic law, which states that ontogeny recapitulates phylogeny. And since the skeleton of a vertebrate embryo is composed at first of cartilage, it followed that the skeletons of ancestral adult vertebrates were similarly constituted.

In recent years both theory and fact have been greatly modified. The biogenetic law has been heavily attacked by such authorities as Garstang (1922) and De Beer (1951). The researches of these and other workers have shown that embryos and larvas evolve no less than adults. The presence of some distinctive anatomical feature in an embryo is more likely to be an adaptation to present developmental needs than a reminiscence of past adult structures. Thus cartilage may simply be an adaptation to the need of the embryo for a skeletal tissue that is capable of rapid interstitial growth (Romer, 1942).

Once this approaches its final size, cartilage would give way to bone. It has, of course, been long known that cartilage is not a precursor of bone. Bone may develop in the absence of cartilage, and never develops from cartilage, though often replacing it.

Modern palaeontological discoveries accord well with such views. The earliest known vertebrate fossils are those of small, heavily armoured, fish-like creatures called ostracoderms, whose remains are found in rocks of Ordovician, Silurian and Devonian age. Thanks to the classical researches of Stensiö (1927), we know a good deal about the anatomy and biology of the ostracoderms. Their skeletons were made of bone, and in some of them the dermal armour was remarkably massive. The earliest known vertebrates possessed true bone, and some of them had a great deal of it.

The internal skeleton of the ostracoderms was sometimes cartilaginous, and sometimes more or less ossified, but the exoskeleton was always bony. It is interesting to note that both endochondral and membrane bone are to be found in the earliest vertebrates, whereas it was formerly believed that membrane bone was an evolutionary novelty of comparatively recent date.

The earliest ostracoderms were rather small, ranging in length from several inches to a foot. Each species had its characteristic size, and examples deviating much from the norm are unknown. It is believed that they passed through a larval phase in which the skeleton was entirely cartilaginous, and when the larva had grown to the adult size it underwent a metamorphosis in which a bony skeleton and dermal armour were acquired. In the same way the brook lamprey has a long larval period followed by a metamorphosis when it is almost full-grown, although it never acquires a bony skeleton. No remains of these hypothetical larval forms have been found, but it is not to be expected that soft, embryonic cartilage would often be preserved. In the sharks, in which the skeleton is permanently cartilaginous, it becomes more or less calcified with age, and skeletal remains of sharks are often found as fossils.

The available evidence, then, suggests that, in the earliest vertebrates, the skeleton was entirely cartilaginous during development, and almost entirely bony in the adult condition, and that once maturity was attained growth almost or quite ceased.

The dermal armour of these early vertebrates consisted of three layers, a thin outer layer of hard dentine, a thick middle layer of vascular bone, and an inner layer of lamellar bone. Dentine, too, is found in the earliest known vertebrates, and it covered the whole exoskeleton. It is interesting to compare this armour-plating with that of modern tanks and battleships. Here the same three layers are employed, a thin, case-hardened outer layer, a softer, but very tough middle layer, and a somewhat harder inner layer.

In the existence of this hard outer layer may perhaps be found one of the reasons why the early ostracoderms never grew after metamorphosis. Ordinary bone grows by external accretion, but dentine is laid down from within, so that the outer surface is definitive. As the armour of the head region consisted of a few relatively large plates solidly welded to the endoskeleton, growth at the sutures seems to have been ruled out.

Some of the heavily armoured vertebrates which immediately succeeded the ostracoderms had lost the dentine layer of the exoskeleton, even the teeth being made of ordinary bone. These fish-like creatures were capable of continued growth, and some of them, the coccosteids for example, attained a very large size, up to 20 ft. in length. At the other extreme, the sharks and rays have lost all bone except the dentine layer, which is represented in the teeth and the spiny denticles. This lack of bone in sharks was once considered to be a primitive feature, and they were, therefore, thought to represent the ancestors of the bony vertebrates. As a matter of fact, sharks are the last of the major groups of fishes to appear in the fossil record, so they are not entirely primitive. Many other groups of fishes show a tendency towards loss of bone, or rather towards its non-development. Thus the modern lung-fishes have very little bone, although the fossil lung-fishes were very well ossified. The sturgeons are completely cartilaginous except for a row of bony scutes along each side. Thus the condition in sharks is not unique.

In the evolution of the bony fishes the exoskeleton tends to break up into a number of small plates or scales, and dentine is generally confined to the teeth. In some of the fossil fishes the dentine layer was retained all over the body, being periodically resorbed to allow the bony skeleton to grow. In others, the so-called ganoid fishes, a few species of which still survive, the outer face of each scale is covered with an enamel-like substance called ganoin, which is laid on from without; thus continuous, concentric growth is possible. As a rule, however, modern bony fishes show a tendency towards reduction in the external armour, the scales become thin and papery, and in many species they disappear altogether. Only the bony plates covering the head remain, but these lose much of their original protective function, serving instead for the support and movement of the jaws, breathing apparatus and pectoral fins. Here the bones never become closely knit together, but preserve a great deal of freedom of motion. The fish skull is not a solid structure like that of a terrestrial vertebrate, but a mechanism of quite surprising complexity. Terrestrial vertebrates show a generally opposite evolutionary trend, the skull tending to become a compact, bony box. Only in birds and some reptiles do the skull elements show some power of relative movement.

Some mention may be made here of the comparative histology of bone. Certain evolutionary trends seem indicated here, although there are still many gaps in the account. The bone of the ostracoderms contained no osteocytes, and this condition is also found in the more advanced of the bony fishes. In these, once a skeletal element has become ossified, further growth is by concentric accretion, and there is little or no formation of secondary bone within. This, and the fact that growth is continuous, make it possible to tell the age of a fish by examination of the cementing lines and growth ridges in suitable bones and scales. This method of age determination would not be possible in a mammal, where growth eventually stops, and the periosteal layers are eventually replaced by secondary bone.

The more primitive of the bony fishes, however, have cellular bone, and this is of some interest, as these fishes are allied to the ancestors of the terrestrial vertebrates. The arrangement of the lacunae is not very regular, and canaliculi are few, sometimes entirely lacking. Even when numerous, the canaliculi form few connexions with those from neighbouring cells. It follows that, as the bone increases in thickness, the deeper osteocytes are gradually deprived of nourishment. Eventually they die and disappear, leaving empty lacunae. Thus the deeper bone comes to consist largely of non-living tissue, rather like the heart-wood of a tree.

The amphibia show, on the whole, a more regular arrangement of the osteocytes, the canaliculi are more numerous, and more interconnected, and there are fewer empty lacunae. The diaphyses of the long bones are composed entirely of periosteal bone, and the osteocytes show a concentric arrangement, as though the diaphysis were a single large Haversian system. In some of the more primitive salamanders, however, the cellular arrangement is hardly more regular than in the bony fishes.

Conditions in the reptiles are interesting, inasmuch as they exhibit a series of intermediate stages in the evolution of the mammalian type of bone structure. In all reptiles the inner part of the periosteal bone becomes excavated and replaced by secondary bone. This replacement is never complete, and as a rule the cellular arrangement in the secondary bone is very irregular. In the tortoise, however, the secondary bone is laid down in Haversian systems. As with other reptiles, replacement is incomplete, cellular arrangement in the periosteal bone is irregular, and there are many empty lacunae (Crawford, 1940).

Finally, mammalian bone is characterized by great regularity in the cellular arrangement. Most of the periosteal bone is replaced by secondary bone arranged in very regular Haversian systems. Osteocytes are well interconnected, and empty lacunae are rare.

So far the evolutionary account seems fairly straightforward; actually there are a good many gaps and contradictions. Mention has already been made of the coccosteids, large fish-like creatures with peculiar jaws and bony teeth, which lived in the Devonian era shortly after the ostracoderms. The head and thorax were very heavily armoured, and this armour shows secondary bone arranged in Haversian systems. Gebhard (1907), who described this condition, only published a preliminary announcement without any illustrations; the complete account which he then promised does not seem to have appeared. Nothing is known of the bone structure of the Mesozoic and late Palaeozoic reptiles and amphibia, and one would think that a comparative study of these would be most rewarding. Up to the present, however, more is known about the evolution of bones as organs than of bone as a tissue.

SUMMARY

The possession of bone is one of the most ancient vertebrate characteristics, and its primary function seems to have been protective. The function of support seems to have been of less importance, since a tendency for the bony endoskeleton to revert to cartilage has appeared in many groups of fishes. The dentine layer, which at first covered the whole of the armour, became confined to the teeth (except in the sharks), the body plating became reduced to thin scales, and even these have disappeared in many species. The plating of the head acquired new functions in connexion with the mechanism of the jaws, breathing apparatus and pectoral fins.

With the appearance of terrestrial vertebrates bone acquired a new importance in connexion with the problem of supporting a heavy body on land. Except in very small animals, cartilage becomes largely a juvenile and embryonic tissue. The central vacui-

ties of the bones become the site of haematopoietic tissue, and in the higher vertebrates bone is known to act as a storehouse of mineral salts.

In its finer structure, too, bone shows a progressive evolutionary change, although here the account is far from complete. In the earliest vertebrates the bone is acellular. In the primitive bony fishes the bone is cellular, although the arrangement of the lacunae is very irregular, and the osteocytes do not long survive. In the advanced bony fishes the trend has been once more toward the acellular condition, but terrestrial vertebrates show a trend toward greater regularity, longer survival of the osteocytes, and the appearance of nutrient canals and secondary bone arranged in Haversian systems. These trends reach their culmination in the bone tissue of modern mammals. There is, however, some reason to believe that some of the most ancient fish-like vertebrates had also a very regular type of cellular arrangement.

Knowledge is lacking of the histology of bone in many extinct vertebrate groups, but the fact that bone structure is known to vary so greatly between the different groups studied suggests that this knowledge would be well worth having. Even less is known about the reasons underlying this variation. The problem of correlating the evolution of bone structure with that of bone function remains one for the future.

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Bone as a Skeletal Structure

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Bone has provided absorbing problems for biologists for many hundreds of years. A fascinating account of the early history of this subject is to be found in *Menders of the Maimed* by Sir Arthur Keith (1919), a new edition of which is to be published shortly. The point of interest has varied from time to time but the basic problem is still the properties of bone as a supporting structure; this is the practical aspect of bone physiology which is most obvious to the man with a fractured femur, or to the surgeon who looks after him. I reviewed the information, such as it is, on the healing of bone some years ago (Bell, 1945) and I propose to confine my attention here to what might be termed engineering problems. These are, firstly, the search for the factors determining the shape of a bone and, secondly, the measurement of the physical properties of bones.

In the embryo the earliest cartilaginous rudiments possess the characteristic shape of the future bone, for example the head, trochanter and condyles of the femur can be recognized; furthermore, femora removed from $5\frac{1}{2}$- and 6-day-old chick embryos, and grown by tissue-culture methods, increase to several times their original length and yet still maintain their characteristic shape (Fell & Robison, 1929). Since in tissue culture factors such as the weight of the embryo, muscle pull, relationship with other tissues and the circulation are absent, there is no doubt that the general form of a bone is to some extent under the control of intrinsic factors. This power of self-differentiation, picturesquely described by John Hunter as a form of consciousness, is still present to a remarkable degree in the human infant after birth. For example, the fragments in a birth fracture of the femur are usually firmly united in 2 or 3 weeks and even if union takes place with the fragments overlapping at right angles the process of remodelling is carried out so efficiently that in less than a year the radiologist finds it difficult to decide whether the right or left femur had been fractured (White, 1944). Even in older children it is remarkable how deformities after fracture eventually disappear.

There is no lack of evidence, however, that the final form of the bone is influenced by factors external to it. In a case of acute osteitis of the radius the bone may have to be removed; the graft from the tibia put in its place quite soon takes on the typical appearance of the radius with a normal medullary cavity (White, 1944). Muscle function undoubtedly has an effect on the shape of the bones. Ross (1950) has reported the case of a labourer who lost all the fingers of the right hand except the little finger. Thirty years later he had a very powerful little finger with great increase in the thickness of the metacarpal bone and of the phalanges. The size of the jaw bones is influenced by the physical composition of the diet. On a rough diet requiring considerable mastication the mandibles of rats are heavier and thicker, especially at the areas of muscular attachment, than they are on a soft pappy diet (Watt & Williams, 1951). The characteristic triangular cross-section of the tibia is due partly to the pressure on it of the anterior tibial muscles. If these muscles are atrophied the tibia has a rounded cross-section like that of the foetal bone (Murray, 1936). We may have here a conflict between extrinsic and intrinsic forces; the intrinsic forces tend to produce a rounded section which might be more efficient structurally, but the extrinsic forces modify this to the angular shape. Washburn (1947) investigated the effect of the muscle pull on the mandible by removing one temporal muscle from newborn rats. Between 3 and 5 months later the coronoid process of the mandible was completely resorbed although at birth it was well formed. The form of the skull was, however, unaffected by the loss of the temporal muscle. The relationship between the growth of the brain and the growth of the overlying brain case is quite mysterious. For example, MacDougall (1951) has described a case of a child of 4 weeks, of normal appearance, whose cranium contained only the basal nuclei and a small portion of the occipital lobes in addition to the cerebellum. The frontal and parietal lobes were one vast cyst with no evidence of increased tension. In spite of the absence of the brain, the brain case was normal. Wolffson (1950) removed various scapular muscles from rats on the 1st or 2nd day of life; the non-operated side served as control. It was found later that the shape and size of the scapula was much changed.

There is considerable evidence of nervous influences on bone growth. An obvious example is the small limb in birth palsy, or the slight diminution of the bones of the paralysed limb in cases of anterior poliomyelitis. The latter effect can be imitated experimentally by cutting the ventral roots of the spinal cord in growing cats (Bell & Robertson, 1946). These lesions are, however, accompanied by paralysis and it may be that the loss of muscle pull, as in Washburn's (1947) and Wolffson's (1950) experiments, is even more responsible than the loss of the connexion with the nervous system for the change in size. This objection cannot, however, be sustained in the description by Penfield & Robertson (1943) of the 'comparative moderate smallness' of the contralateral part of the body in children with lesions of the postcentral gyrus, even when the precentral gyrus is normal. The alteration in size produced by such cortical lesions is, however, much smaller than that seen in spinal lesions. The atrophy following nerve section can sometimes by overridden. Wermel (1934, 1935) found that, in rats, complete or partial removal of one forearm bone was followed by an increase in the thickness of the other. No doubt this operation increased the stress on the remaining bone. This, however, is not the full explanation, since the remaining bone increased in size even when the functional activity of the limb was reduced considerably by nerve section.

The pattern of the cancellous tissue in long bones has provided the anatomist-cum-engineer with most intriguing problems. The cancellous tissue in the head of the femur is especially well developed and undoubtedly the spongiosa is distributed to resist the stresses to which the neck of the femur is subjected. The head, neck and shaft of the femur have been compared to a crane in which two stresses have to be allowed for, namely tension and pressure. An economically constructed crane is designed so that the greatest mass of material is placed where the stress is greatest. In the trajectorial theory the cancellous tissue has been picturesquely described as being a crystallization of the lines of force. If this is so, the bone tracts or trajectories of pressure and tension should cross at right angles. This is not always so, and in any case the pattern varies considerably from one specimen to the next. Jansen (1920) points out that in coxa vara, although the tension in bearing the weight of the body is greatest on the convex side of the neck, the strongest development of the spongiosa does not occur there but on the concave side where there is pressure. He believes that pressure is the adequate stimulus for bone formation. Tension in tendons, aponeuroses, fasciae and ligaments does not induce ossification. It is clear, however, from tissue-culture experiments that bone can form in the entire absence of pressure or tension. We have, moreover, no idea as to how stresses in bone act on osteoblasts or osteoclasts. Furthermore, such static considerations do not do justice to the full function of the femur. The muscles arising from bones probably apply to them greater forces than does the fraction of the weight of the body that is supported by the bone. Indeed it has been calculated (Bell, Cuthbertson & Orr, 1941) that bones are very much stronger than would be necessary if they were mere props. They have been designed by nature for much more severe stresses.

I should like to mention briefly two techniques recently applied to whole bones. The first is the method of estimating bone density painstakingly worked out by Mack, Brown & Trapp (1949) and Mack (1950, p. 30). An X-ray photograph is taken of a bone together with an ivory step wedge. From this their microphotometer can produce a graph of the amount of mineral matter at any cross-section of the bone, such as at the hand, the foot, or the knee. They have found that the ends of a long bone, such as the femur, and also the os calcis change rapidly in density when the calcium intake is altered, whereas the phalanges are very slow to change. Here at last is a method for investigating the relation between nutrition and bone structure in man. Interesting results may be expected from the surveys which Mack is now carrying out.

The second is the stress-coat deformation technique used by Evans and his colleagues (Evans, Pedersen & Lissner, 1951). Bones from cadavers are sprayed with brittle lacquer (originally used to test aircraft structures) and are then loaded by a hydraulic testing machine. At a certain tensile strain the lacquer cracks and the cracks, when filled with indian ink, are easily photographed. It has been shown that tension is the main cause of failure, that is fracture, of bone.

If one believes that it is an advantage to possess strong bones then two problems arise—how is the strength of the bones to be measured and what are the nutritional factors likely to bring about this strength? With these questions in mind we (Bell *et al.* 1941) tried to find the effect of various levels of calcium intake on bone growth and strength. Growing albino rats were put for 2 months on a diet of constant protein, carbohydrate, fat and vitamin content but of varying calcium content. No matter whether the calcium content of the diet was very low or very high the external dimensions of the femora were almost the same. On the very low calcium intakes the bones formed had very thin walls; as the diet improved up to 0·36% calcium so did the femoral wall thicken and the marrow cavity diminish. Greater calcium consumption made no difference whatsoever to the bones. The strength of the bone measured by bending tests (Bell *et al.* 1941; Weir, Bell & Chambers, 1949) showed that, whereas the strength of the individual bones increased with increased calcium intake up to this same point, the breaking stress, which measures the quality of the material, was the same in all experiments. In other words, if one compares the shaft of the femur to a factory chimney, it appears that, on a good diet, varying only in its calcium content, the quality of the bricks throughout is of the highest and the external appearance is the same. The only variation is in the number of bricks laid down to form the thickness of the wall. The central space cannot for some unknown reason be encroached on to more than a fixed extent.

The picture is, however, very different when the basic diet is defective and especially when it is so defective that rickets is produced (Bell, Chambers & Dawson, 1947; Weir *et al.* 1949). In these experiments young rats were placed on a diet with a very abnormal calcium:phosphorus ratio (5:1). Litter-mates received the same diet supplemented with vitamin D, and other litter-mates received a good stock diet. The growth of the animals on the rachitogenic diet was very poor and the addition of vitamin D did not increase the rate of growth very much. The bone growth in the three groups, as shown by the size of the bones, varied much in the same way as the body-weight. The ash content of the bones produced on the rachitogenic diet was very low indeed (30%). The ash content was higher (40%) in those given cod-liver oil and reached its usual maximum value (60%) in those on the normal stock diet. In all instances, however, the calcium:phosphorus ratio in the ash was about 2. The individual bones on the poorest diet were much weaker and the breaking stress showed that, on the factory-chimney analogy, the quality of the bricks was very poor. Rickets in rats can be produced only by grossly abnormal diets; from the dietary point of view rickets in dogs is more like human rickets. We examined some rachitic puppy bones kindly supplied by Lady Mellanby and found that the ash content and the breaking stress were similar to those found in the rats. There was in the rats an increase in Young's modulus of elasticity as the diet improved. The best way to compare the three lots of bones is by stress-strain diagrams which, since the influence of the size of the bone has been eliminated by calculation, allow direct comparison of the bony material in each group. The breaking stress and Young's modulus have been found to be very closely correlated, and since Young's modulus, unlike breaking stress, can be measured without damage to the bone, it may be that here we have a hint of a method which might be applied to living bone material. So far as we can tell at present the diminution in breaking stress and Young's modulus in rickets is due to the decrease in the amount of ash in the bones and is not associated with any chemical or structural alteration.

Normal bone is a remarkable material. The average breaking stress from our tests in bending is approximately 30,000 lb./sq.in. which is nearly as good as for cast iron. Young's modulus is, however, only one-tenth of that of cast iron. Thus bone, despite its lightness, is remarkably strong and its relatively great flexibility helps it to absorb sudden impacts.

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Growth of Bone

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The plasticity of bone is generally recognized and bone is sometimes given the credit of being second only to blood in this quality. During growth its plasticity is especially apparent.

The ossified diaphysis of a humerus of a 5-month-old foetus is a tiny miniature of the adult, with a bony diaphysis of about 35 mm, a girth at the middle of less than 3 mm and cartilaginous ends. This foetal bone, when placed within the medullary cavity of the adult bone, is almost lost, but if its life and growth had continued, it would have created within itself a medullary cavity many times its own original size and formed a shaft and ends that would have retained the same essential form throughout its foetal months and postnatal years (Pl. 1, 1).

The cartilaginous precursor of a long bone early assumes a form that embodies the main features of the adult bone and so each has its individuality stamped on it. In any consideration of bone growth this early assumption of adult form and its retention throughout the period of growth must be borne in mind and the problems pertaining to bone growth can never be dissociated from this conservation of shape.

Over two hundred years ago Stephen Hales (1727) deduced from a single experiment that bones grow at their ends and not interstitially. Punching two holes half an inch apart in the shaft of the tarso-metatarsal bone of a young chicken, he observed that 2 months later they remained separated by the same distance although the whole bone had grown an additional inch; he furthermore noted that the greater part of the growth had occurred at the upper end of the bone. The account of these observations is preceded by a reference to the growth of cartilage which he describes as the non-ossified 'tender ductile bone of young animals'. Growth in this tissue he postulates as occurring in every part, and this may justifiably be interpreted in our terminology of to-day as interstitial growth. Adequate credit should always be given to the observations of Hales on both cartilage and bone as they are the first record of the manner in which cartilage grows in size and long bones grow in length, and the truth of them has been upheld by the many others who have investigated this problem; the fact that one experiment only was conducted should not detract from the value of Hales's accurate observations and deductions. The celebrated researches of Duhamel (1743) and Hunter (1837) on the growth of long bones in part at least constituted an elaboration of Hales's earlier work, but they carried the problem a stage further by their inquiries into the growth in girth of long bone. John Hunter, in his investigation of this phenomenon, utilized an observation that had been recorded by John Belchier in 1736, namely that pigs fed with a diet containing an infusion of madder root had certain parts of their bones coloured red. Hunter demonstrated that the portion of bone stained by the madder root was restricted to that portion of the bone laid down during the feeding of the dye, the remaining part retaining a normal bone colour. By continuous and intermittent feeding of the dye, Hunter conclusively proved that bones grow in girth by the deposition of new bone on the surface, and that the simultaneous widening of the marrow cavity occurs by the absorption of the bone lining it. Hunter also directed his attention to the growth that occurs in the neck of the femur, a part of the diaphysis, and observed that during growth there is an accretion of bone on its upper aspect and an absorption of bone from the lower so that the form of the neck is retained. Brash (1934 *a*, *b*) in his meticulous studies on the bones of madder-fed pigs has analyzed the growth of some of the skull bones and of the mandible and his observations have proved without doubt that bone growth and bone absorption are solely surface phenomena. The definitive form assumed by any bone is therefore determined by the synchronous deposition and absorption of bone, and any major upset in the balance of these normally harmonious processes will in turn reflect itself in a departure from normal in the form of a bone.

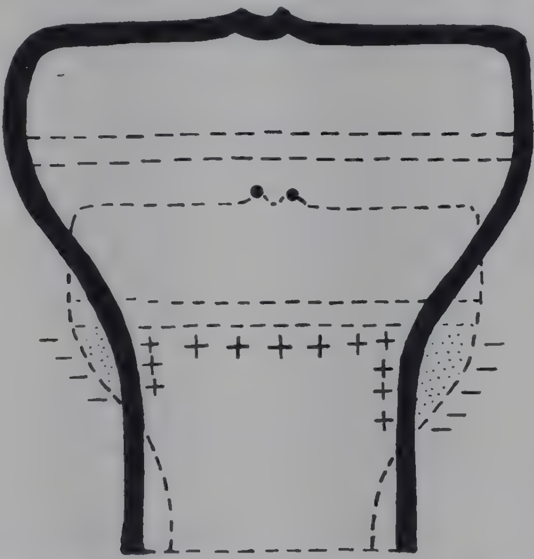

Fig. 1. Schematic diagram of the growing end of a long bone to illustrate the modelling resorption on the outer surface of diaphysis (indicated by minus sign) and the deposition of bone on the endosteal surface (indicated by plus sign). The absorbed bone has been stippled. The region of the diaphysis, at which growth in length takes place, is also indicated by plus signs.

Lacroix (1951) and Leblond, Wilkinson, Bélanger & Robichon (1950) have drawn especial attention to the modelling of bone that occurs in the diaphysis adjacent to the epiphysis, and the latter workers, who used radio-phosphorus ^{32}P in their investigations, have stressed that the ossification that occurs in this region is endosteal in type; they have, moreover, emphasized that their conclusions are in complete accord with the observations of Kölliker (1873). (See Fig. 1.)

No account of growth of bone, however abbreviated, can fail to include reference to the observations of Harris (1933) on the lines of arrested growth that may be seen by radiography in the bones of young people who have suffered from acute illness. These lines, which are semi-permanent, appear at both ends of the diaphysis and as long as they persist they remain the same distance from each other throughout the growth of the bone, although they recede at unequal rates from the ends. This feature of the lines of arrested growth is to be regarded as additional proof that growth in length of a bone occurs only at the ends and not interstitially.

In this attempt to summarize the mode of growth in girth of a bone it has been stated that this process is essentially a surface phenomenon, and by this it is implied that new bone is always laid down on the surface of old bone. It is at this stage that I should like to extend this conception of growth to the growing ends of bone as in these situations new bone is formed on the surface of cartilaginous remnants or on bone already formed so that the process there again is truly a surface phenomenon. As the conception of surface growth in these situations is perhaps more difficult to appreciate, the term of appositional growth as favoured by Weinmann & Sicher (1947) is perhaps preferable. All bone growth, whether in relation to growth in girth or growth in length, is therefore regarded as appositional growth, and this concept is in accord with all known facts.

A necessary prerequisite to an appreciation of bone growth is a familiarity with the early stages of ossification, and in those bones that are preformed in cartilage this in turn hinges on a knowledge of the formation of their cartilaginous forerunners. The consideration of the early stages of chondrification of the humerus and its subsequent ossification formed one of the last works of that master of embryology, George L. Streeter (1949). In the 5 mm human embryo the limb buds are established as outgrowths from the non-segmented part of the body wall and each is first represented by a surface layer of ectoderm, and an undifferentiated mass of mesenchyme; evidence of the skeletal condensation can be well seen in the 12 mm embryo. Thereafter the cartilaginous model rapidly assumes the general form of the bone which is to replace it, and the determination of this form must lie in the chondroblasts that lie in the deeper layer of the perichondrium. It is the multiplication and growth of the chondroblasts and the formation of intercellular substance that determine the shape of these primary skeletal elements.

The changes that occur in the oldest cartilage cells in the central region of the cartilaginous model are well known, and it need only be stressed that one can trace successive phases of younger cells as one passes to the ends of the cartilaginous model (Pl. 1, 2).

Bloom & Bloom (1940), in their consideration of the calcification of developing bone, have shown that calcification of the cartilage always precedes its vascularization. At this time the cartilage matrix in the middle of the shaft is represented by thin laminae between the lacunae that lodge the degenerating cells and it is in these laminae that the calcification occurs (Pl. 1, 3). Simultaneously with the calcification of the cartilage a perichondrial collar of bone is formed around the central part of the shaft so that the first ossification that occurs in a cartilaginous bone is of the membranous type (Pl. 1, 4). This collar of bone, as stated by Streeter (1949), is a continuous collar and does not in the first instance show the vascular foramina that are such a prominent feature of it at a slightly later date.

The inductive influence of the central cartilaginous cells in the formation of the perichondrial osseous collar has been fully presented and discussed by Lacroix (1951), and Streeter (1949) suggests that the stage of degeneration which these cells attain is the apotheosis of their development.

The entirety of the osseous collar is soon disturbed by the appearance in it of lacunae through which osteoblasts stream and blood vessels pass. The breakdown of the cartilage is thereafter relatively great and it is on spicule-like remnants of it that endochondral ossification occurs (Pl. 1, 4, 5). These cartilaginous remnants are gradually replaced by bone or disappear without replacement, and as the ossific process extends towards the ends of the cartilage and the bone gains in width by subperiosteal growth, the earliest formed bone is absorbed and the medullary cavity thereby increased in size. It is not appropriate for me to enter into a discussion of the part played by the osteoblast or the osteoclast, but the microscopical studies of Kölliker (1873) on the last-named cell have frequently been substantiated by other workers, and the sites of its presence have been repeatedly verified as the sites of bone absorption. (See also, Leriche & Policard, 1928; Arey, 1920; Dodds, 1932; Leblond *et al.* 1950).

In many long bones an epiphyseal centre or centres of ossification appear within the cartilage at both ends, and these differ from the diaphyseal process of ossification in being entirely endochondral. It is generally recognized that the advent of ossification in the cartilaginous ends bears no relationship to the volume of the cartilage, as a well-developed system of vascular canals is clearly demonstrable in the cartilage ends of all the larger long bones before ossification (Pl. 2, 5). The character of these canals has been fully discussed by Haines (1933) and Hurrell (1934).

In man the secondary centres of ossification appear throughout the first 15 years of life, and though the time of appearance of any one particular centre may differ in different individuals over a period of several years, the sequence of appearance of secondary centres is remarkably constant. The time of the appearance of a secondary ossific centre bears some relationship to its time of fusion and in any one bone the centre that appears first usually fuses last (Pl. 2, 1–4). For this reason a long bone has usually one end which contributes more to the length than the other, but this depends less on the longer life of the epiphyseal or growth cartilage at the growing end than on the greater activity of the cartilage at that end (see Payton, 1933; Harris, 1931*a*, *b*; Gill & Abbott, 1942). In the humerus, for example, about 80% of the total elongation is estimated as occurring at the upper growth cartilage (Bisgard & Bisgard, 1935), and in the observations that I have made of the vertebrae of various mammals I have been able to correlate the greater activity and the longer life of the growth cartilage that separates the caudal epiphysis from the vertebral body.

The definitive form of bones is regarded as dependent partly on the growth pattern inherent in the bones themselves and partly on the extrinsic forces to which they are subjected throughout life. Murray (1936) has presented a most admirable survey of his own investigations and those of others on experimental work in embryos that has proved the self-differentiating qualities of limb bones. The report of Murray & Huxley (1925) on the result of grafting the hind-limb bud of a 4-day chick embryo on the chorio-allantoic membrane of another chick was a major contribution to the knowledge of bone growth. After 6 days' cultivation the undifferentiated mesenchyme of the bud had formed a recognizable femur with a collar of perichondral bone. The inherent power of differentiation may be demonstrated in the human femur, where a patellar surface develops with the characteristic larger lateral patellar lip that is so essential for the normal retention of the patella in the extended position of the knee joint; this asymmetrical development of the patellar surface occurs when the patella is not in contact with it, owing to the extreme flexion of the knee joint. In contra-distinction to this inherent power of self-differentiation, it is of interest to note that the tendon of the popliteus which is so closely related to the lateral femoral condyle throughout foetal life stamps a groove that persists throughout adult life.

In this synopsis of some aspects of bone growth, I have knowingly omitted much that is of paramount importance in the subject, but to do otherwise in the space at my disposal would be quite outside my power.

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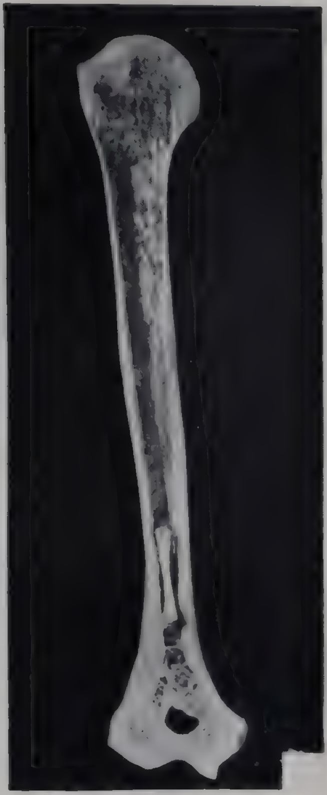
EXPLANATION OF PLATES

PLATE 1

1. Photograph of section of the adult human humerus in the medullary cavity of which lies the diaphysis of a 5-month foetal humerus. About 80% of growth in length of the humerus occurs at the upper epiphyseal cartilage, hence the foetal humerus has been placed near the lower end of the adult bone. × ½.
2. Longitudinal section of human foetal phalanx showing degeneration of cartilage cells in mid-zone before formation of bone. × 68.
3. Section of human foetal phalanx showing further stage of degeneration of cartilage cells in mid-zone. Lacunae containing cells are separated from one another by thin calcified laminae. Perichondrial collar of bone surrounds degenerating cells and is seen as dark band between cartilage and perichondrium. × 56.
4. Section of human foetal phalanx after appearance of primitive medullary cavity. Periosteal collar of bone is now clearly evident and mid-zone of cartilage has been absorbed. × 17.
5. Mid-zone of same phalanx as in 4, showing vessel piercing periosteal collar of bone: bone is also being laid down on remnants of calcified cartilage. (The bone is more darkly stained than cartilage.) × 87.

PLATE 2

1. Sagittal section of intervertebral disk and parts of adjacent vertebrae of young rabbit. The epiphyseal (or growth) cartilage is seen separating the diaphysis of the vertebral body from the epiphysis in both the upper and lower vertebrae. Most of the growth in length of a vertebra occurs at its lower end and therefore the epiphyseal cartilage in the upper vertebra is more active than that in the lower. × 12.



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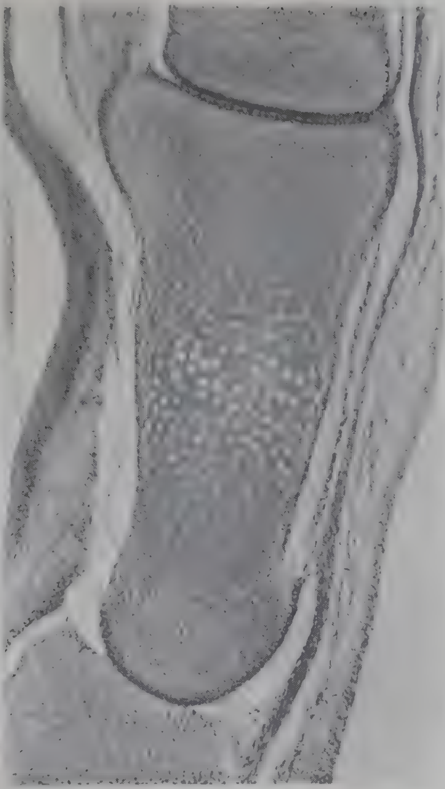

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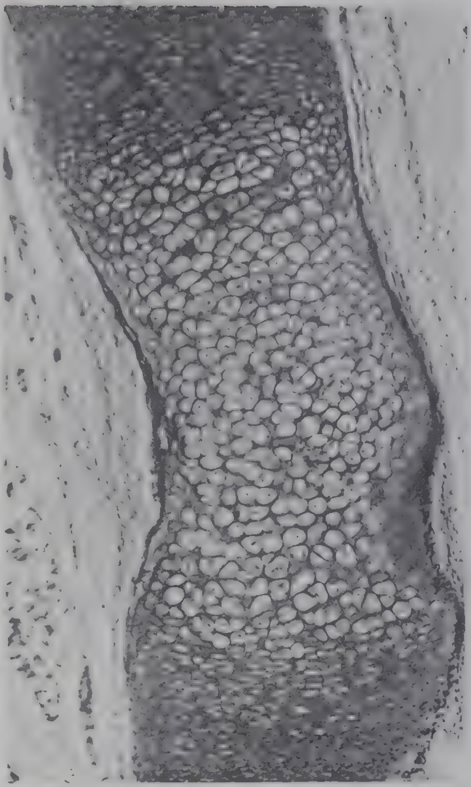

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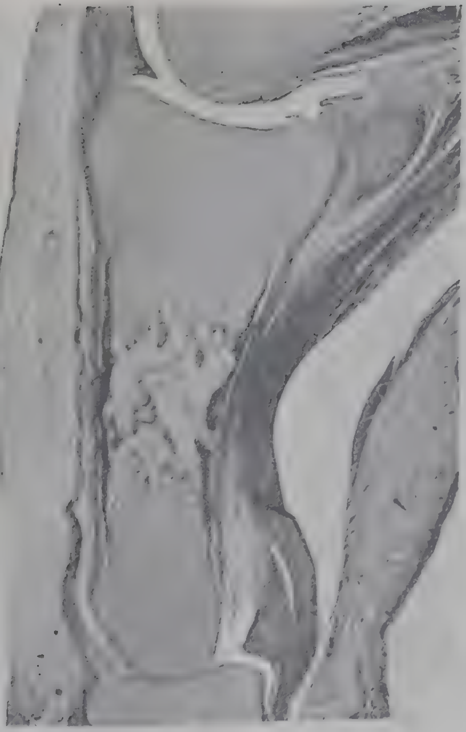

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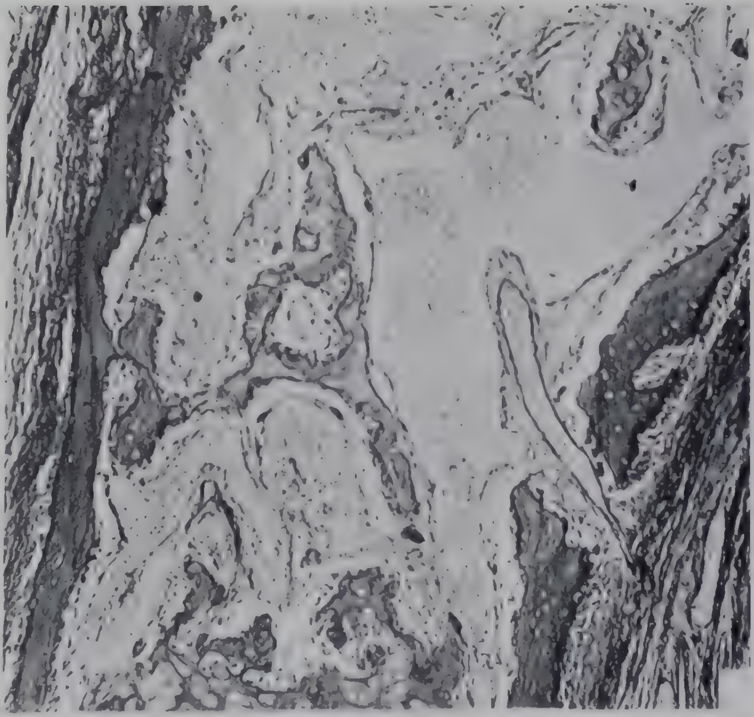

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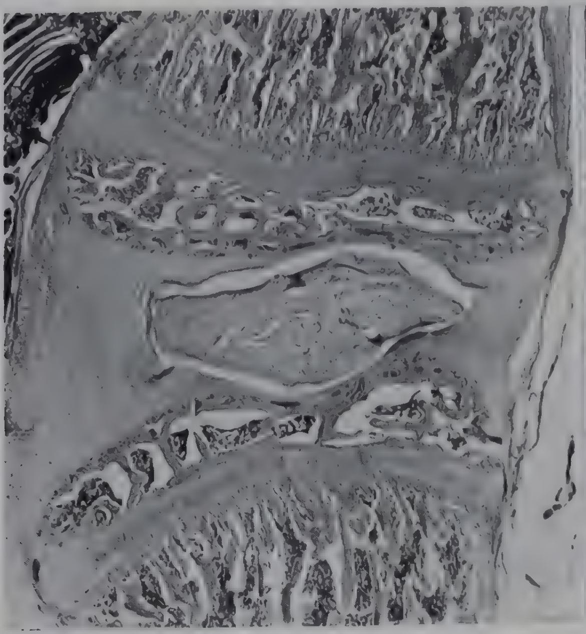

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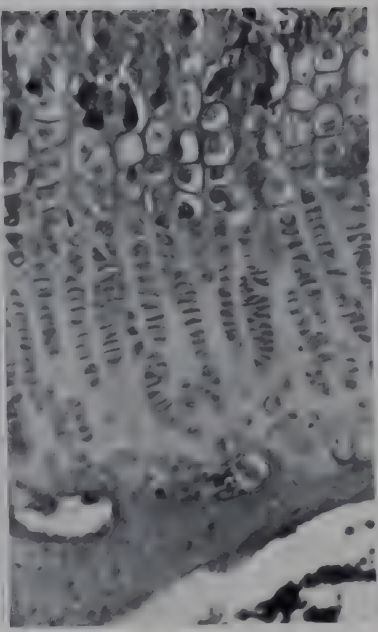

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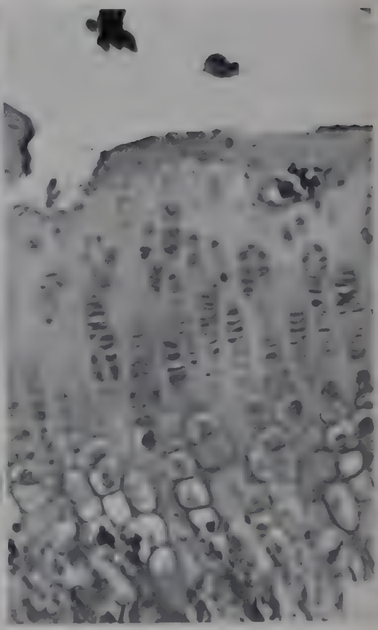

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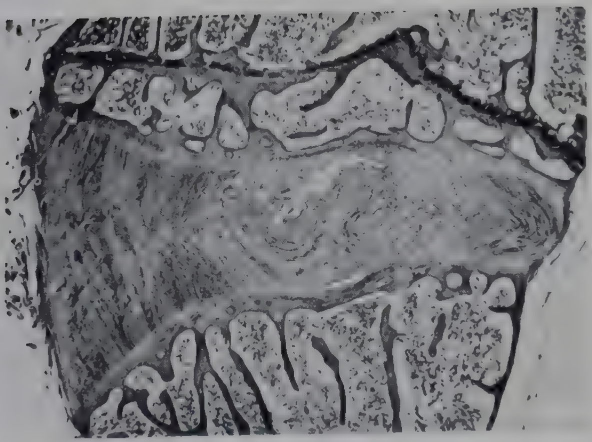

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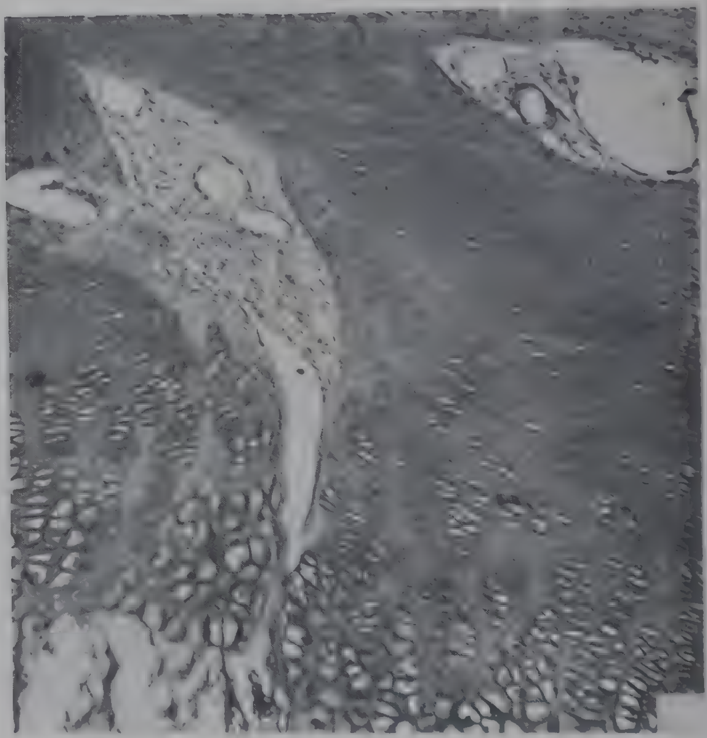

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2. Part of epiphyseal cartilage of upper vertebra shown in 1. Long columns of cartilage cells, indicating the intense activity of the cartilage, are evident. × 130.
3. Part of epiphyseal cartilage of lower vertebra shown in 1. Cartilage columns are shorter than those in 2, indicating less marked activity of the growth cartilage. × 130.
4. Sagittal section of lumbar vertebrae of mature rabbit showing persistence of epiphyseal cartilage in upper vertebra. This figure demonstrates that the more active epiphyseal cartilage in a bone tends to persist longer. The lower end of a vertebral body in most pronogrades is the 'growing' end. × 10.
5. Section of upper end of femur of newborn child. The ends of most long bones before ossification are nourished by vessels running in cartilage canals. Some of the vessels pass from the diaphysis into the cartilaginous end and an example of this is seen in this figure. The cartilage is in the upper part of the figure and the diaphysis of the bone towards the lower part. × 86.

Bone as a Mineral Reserve

By E. C. Owen, *The Hannah Dairy Research Institute, Kirkhill, Ayr*

Bone gives its possessor biochemical as well as structural advantages. Bone is not a dead tissue and the calcium and phosphorus which give it its hardness are also labile, if need be as a source of nourishment for the softer more actively metabolizing tissues of the body. Ca deficiency in wild animals should be a rare phenomenon. P, however, is much harder to come by. Fishes living on the continental shelves of the various land masses acquire P by eating small arthropods and algae. The small plants and animals of the phyto- and zoöplankton keep the phosphate wastes from the land masses in biological circulation. The fate of P has, in the past, been to pass from the fish to the birds which prey on them and so to be deposited on oceanic islands as guano. Rock phosphate deposits, on which modern agriculture, and therefore human nutrition, largely depend, are chiefly fossilized guano. Two very large deposits of apatite, one in the Kola Peninsula, the other in Canada, are exceptions in being igneous in origin. As every farmer knows P is dearer than he would like it to be. Ca, on the other hand, is literally 'dirt cheap'. It is perhaps therefore not surprising that P deficiency is still a hazard of both plant growth and animal production.

It is a fact of history that all the newer countries had difficulty in raising cattle. The reason was a mystery until Theiler (1919) (cf. Theiler & Green, 1931–2) working in South Africa, showed that the difficulty was due to aphosphorosis. This discovery, together with similar work elsewhere in the world, gave the needed impetus to the superphosphate industry which converts rock phosphate to a form more readily available to plant roots. Nevertheless, for human and farm animal alike, aphosphorosis is still an ever-present hazard. However, if children can get plenty of milk, there is little need to worry about their intake either of Ca or of P. Drinking half a pint of cow's milk per day makes an adult safe from aphosphorosis. An expectant or nursing mother ought to have twice as much. Herring, if well cooked, provide not only both Ca and P in the bones, which one eats, but also vitamin D in the body oil. Though it is to be admitted that low-Ca rickets certainly exists (Skill & Kay, 1934), we still do not know if rickets in children and its counterpart, osteomalacia, in later life are primarily due to a deficiency in Ca or in P. From animal experiments, such conditions could be due to deficiency of either or of both or to an excess of either. Human dietary surveys, backed by an estimation of balances, could decide this point (Owen, Irving & Lyall, 1940). Perhaps the reported effects of sodium phosphate in increasing physical endurance are really due to the disclosure of a partial deficiency of phosphate and of a low level of labile P in the bones. During the course of evolution one can imagine that a well-mineralized skeleton of true bone proved better than cartilage in that a bony skeleton resisted bending, and was superior to a framework of calcium carbonate since bone is more resistant to fracture, and is superior to both these since true bone is a labile substance serving as a P bank from which drafts may be made in times of shortage. Overdrafts will eventually break the bank and cause rickets in infants and osteomalacia or osteoporosis in adults. Such overdrafts may be rendered necessary by a winter-time shortage of P or they may be due to some physiological stress such as pregnancy, lactation, egg laying or even rapid growth on food deficient in bone-forming minerals but adequate in other respects. All these stresses have been demonstrated in well-controlled experiments with laboratory animals (Fairbanks & Mitchell, 1936; Conner, Kao & Sherman, 1941; Zucker & Zucker, 1946; Doyle & Porter, 1951). The clinical literature abounds in records of unfortunate patients who have failed, for dietary or hormonal reasons, to surmount the hazards to the skeleton that result from pregnancy, lactation or growth on diets deficient in minerals (Hunter, 1930*a–c*; Alwens, 1919; Dalyell & Chick, 1921; Hume & Nirenstein, 1921; Park, 1923; Hess & Unger, 1918; Owen *et al.* 1940; Gaunt & Irving, 1939).

There is plenty of evidence in the scientific literature for the hypothesis that, in the metabolism of vertebrates, especially during growth, there is competition between the skeleton and the actively metabolizing soft tissues for dietary Ca and P (Fairbanks & Mitchell, 1936; Zucker & Zucker, 1946; Doyle & Porter, 1951; Conner *et al.* 1941). There seems to be similar competition for dietary magnesium (Duckworth, Godden & Warnock, 1940; Duckworth & Godden, 1941, 1943). Rapid growth is accompanied by poor skeletal mineralization and vice versa.

In this competition the soft tissues are more successful so that, when Ca or P or Mg is supplied by the diet in insufficient amount, or when some other substance in the food, or some pathological or experimental condition, interferes with absorption, the soft tissues take priority over the bones for the supply of these minerals. In a growing animal the result is a poorly mineralized skeleton (Zucker & Zucker, 1946) with denuded trabeculae in the long bones (Hunter, 1930*a–c*). In this competition the organic matrix of the bone acts like the other soft tissues (Zucker & Zucker, 1946). If the stress continues long enough rickets may result in the growing animal. In the adult body osteomalacia, osteoporosis (Dalyell & Chick, 1921) or *osteitis fibrosa cystica* (Hunter, 1930*a–c*) may be the result. Hazards that demineralize the adult skeleton are deficient diets (Dalyell & Chick, 1921), especially if accompanied by frequent pregnancies as in the Chinese women studied by Maxwell (1935), idiopathic steatorrhoea, hyperparathyroidism or exophthalmic goitre (Hunter, 1930*a–c*). Frequent resort to such purgatives as magnesium sulphate (Meulengracht, 1938) or the ingestion of fat of too high a melting-point (Chanda, 1949) deplete the mineral content of the skeleton. In all these instances the soft tissues draw minerals from the skeletal stores until the bones are dangerously depleted. A danger signal may be given by attacks of tetany (Dalyell & Chick, 1921) due to the lowering of the amount of ionized Ca in the blood when the demineralization is acute. Another warning is the bending or fracture of a long bone. Older people tend often to take diets poor in bone-forming elements (Owen, *et al.* 1940). Grauhan & Schulz (1939) showed, that the incidence of fractures increases with age. Hume & Nirenstein (1921) found that the incidence of osteomalacia in time of war increased markedly with age. Even a normal physiological process may be a hazard. It is generally agreed that the intake of Ca, of P and of vitamin D should be increased during pregnancy and lactation. During pregnancy a woman on an adequate diet stores as much Ca and P in her skeleton as she does in the foetus and adnexa (Hummel, Sternberger, Hunscher & Macy, 1936). When the baby is born and she feeds it at the breast these stores are rapidly transferred to the milk. So great is the avidity of the mammary gland for Ca and P that a negative balance of Ca is regarded as normal in early lactation and cannot be made positive by dietary means. Evidently the rapidly metabolizing mammary gland, like the muscles, can successfully compete with the bones for P and Ca.

In the lactating cow, subjected to thyroxine treatment, the mobilization of Ca and P from the bones is so fast that all the Ca in the milk can come from the bones and, at the same time, the loss of Ca in the faeces exceeds the total intake of Ca (Owen, 1948). Under these conditions the P:Ca ratio in the milk increases (Owen, 1948), so that more Ca will be taken from the bones than is required for milk secretion. This extra Ca appears in the faeces. In the same experiment Owen (1948) also found that the cows were in positive P balance all the time and that thyroxine increased the positivity of the P balance. Thus these cows, in depleting the skeletal stores of Ca and P in which the ratio of Ca to P is somewhat more than 2:1, to make milk of Ca:P ratio 1:1, transferred the P to the milk and threw away the Ca. The reverse state of affairs is found in the hen in heavy lay which has to find a large excess of Ca over P to make the shells of the eggs which are almost pure $CaCO_3$. Such hens draw heavily on their skeletal trabeculae but get from that source more P than is needed to make the egg. The excess P is excreted and such hens are in negative P balance (Tyler, 1948–9, 1949–50). Just before coming into lay a hen resembles an expectant mother (Tyler, 1948–9, 1949–50; Hummel *et al.* 1936) in being able to store Ca and P very efficiently in its skeleton. In this instance also, circulating oestrogen is believed to be the cause of the efficient retention. It is likely (Chanda & Owen, 1952) that negative balances of Ca in lactating animals and of P in laying hens are due to a natural increase of thyroid activity in both these physiological states. Teeth do not seem to participate, even during growth, as actively as bone in the body's commerce in Ca, P and Mg. They are therefore protected, to some extent, against hazards to which bone is exposed. This was demonstrated for Ca and P by Gaunt & Irving (1939, 1940–1). Nevertheless, these authors showed, in the same experiments, that rachitogenic diets enhance the predentine width of rats' incisors. Duckworth *et al.* (1940), Duckworth & Godden, (1940–1, 1941, 1943) and Irving (1940) showed that Mg behaved in similar fashion.

Thus, to some extent, maintenance of the composition of the bones and maintenance of the composition of the soft tissues are in conflict. Diets that have an unfavourable Ca:P ratio, or too small a content of either or of both of these minerals, may allow growth of the soft tissues to proceed while the bones and, to a much less extent, the teeth are inadequately supplied with minerals. The result is rickets which clinicians often say improves when children are physically ill. This paradox simply means that, in rickets, the soft tissues take priority over the bones for P and Ca, so that any check to the metabolism of the soft tissues makes P and Ca available for the formation of bone.

In rats, rabbits, pigs, cows, dogs and man it has been shown that bone formation and the mineral content of the bones are adversely affected by diets in which the ratio of Ca to P is greatly different from unity (Shohl, 1939). Vitamin D induces normal formation of bone and tooth when taken with diets which in its absence would produce rickets. The beneficial action of vitamin D is quite general, and all mammals and birds seem to be able to utilize Ca and P more efficiently for bone formation in its presence. When sunshine acts on the skin it is believed that it turns 7-dehydrocholesterol into vitamin D_3. This apparently is the only dietary factor that man can produce by his own photosynthesis. There has been much discussion concerning the mode of action of vitamin D in the body. Experiments with radioactive Ca and radioactive P certainly show that one of the chief actions of vitamin D is to favour the transfer of P from the soft tissues to the bones. This results in retention of Ca along with the P (Cohn & Greenberg, 1939; Gaunt, Griffith & Irving, 1942; Nordfeldt, 1949; Migicovsky & Emslie, 1949). Even before the introduction of the radioactive-tracer technique dietary experiments by Shepherd & Macy (1939) with schoolchildren showed that adding vitamin D to a diet improved the retention of Ca but decreased the retention of S, N and P, just as would be expected if the action of vitamin D were to favour bony- as against soft-tissue anabolism.

In children, in dogs and in cats, but not in ruminants, a diet rich in cereals predisposes the animals to rickets in the growing period (Mellanby, 1921, 1925, 1949). Mellanby postulated the presence of a rachitogenic factor in cereals. It is now known that Mellanby's rachitogenic factor is a Ca-Mg salt of phytic acid. Phytic acid is inositol-hexaphosphoric acid, and its fate in the gut is still a matter of controversy (McCance, 1949). Vitamin D can counteract the action of phytate (Harrison & Harrison, 1951; Singsen, 1948) probably because it increases the efficiency of absorption of that part of the Ca that is not in combination as calcium phytate. The increased efficiency of absorption is probably an effect secondary to the favouring of bone anabolism by vitamin D.

The hormones of the thyroid and of the parathyroid glands are known to affect the transport of Ca and P to and from the bones. The rate of secretion of these hormones by the body may be affected by diet, and the effects of their over- or underproduction are often preventable by dietary means.

In toxic goitre there is a rapid loss of Ca and P by the gut, and Ca balances in such patients may become markedly negative (Hunter, 1930*a–c*). This condition may be simulated in experimental animals of any species by administration of thyroxine. In this type of goitre dangerous demineralization of the skeleton results unless measures are taken to combat the condition. These measures may be surgical removal of some of the thyroid gland or treatment with some such antithyroid drug as methyl- or propylthiouracil.

Rapid demineralization of the skeleton results from overactivity of the parathyroid glands (Hunter, 1930*a-c*). Patients who for any reason have to have their parathyroid glands removed are subject to attacks of tetany. Anderson & Lyall (1939), in Aberdeen, made a very careful study of such tetanies and showed that they can be controlled by increasing the ratio Ca:P in the diet, thus confirming the earlier observations made on parathyroidectomized rats by Shelling (1932). Nowadays, dihydrotachysterol, taken by mouth, enables subjects with defective parathyroids to eat normal diets. Tetany in animals, as in human beings, is induced when the ratio of various minerals to one another in the blood is disturbed. Particularly potent causes are reductions in the concentrations of Ca or of Mg in the blood.

An interesting compensatory mechanism is found in rickets and in osteomalacia. The parathyroid glands are enlarged and active and so ensure that a level of Ca in the blood sufficient to prevent tetany takes precedence over an adequately mineralized skeleton. For a deficiency of vitamin D lasting, say, from one summer to the next, such a compensatory action by the parathyroid glands must, in the evolution of vertebrates, have had a great survival value; but in our so-called civilized conditions such deficiency may continue for longer than one winter so that we go on taking drafts from an already depleted Ca bank till exhaustion of it leaves no protection against attacks of tetany.

Lactose has a favourable effect on Ca retention but other carbohydrates are neutral in this respect. The effect may be due to the fermentation of lactose to lactic acid in the gut, for many organic acids occurring naturally in food, e.g. citric acid and tartaric acid, favour Ca retention on diets that, in their absence, might produce rickets. Citric acid is a normal constituent of body fluids and, according to evidence quoted by Best & Taylor (1950), its secretion in the urine parallels that of Ca. Oxalic acid, which occurs in rhubarb and spinach, interferes with Ca absorption, as it precipitates Ca in the gut as the very insoluble calcium oxalate (Schmidt-Nielsen & Schmidt-Nielsen, 1944).

If an animal is changed from a diet poor in Ca and P to one rich in these elements, its Ca and P balances will become markedly positive (Rottensten, 1938). Even the aged human skeleton, depleted by years of low mineral intake, can store these minerals readily if the patient is given a suitable diet. Adams, Boothby & Snell (1935–6) demonstrated these effects in elderly subjects. The findings of Owen *et al.* (1940) lend support to the theory of Fairbanks & Mitchell (1936) that human Ca balances indicate the degree of depletion of skeletal stores. They showed that the positive Ca balances of some old people were linear functions of the Ca intake. Other aged patients did not show this phenomenon. Presumably the latter were in Ca equilibrium. The experiments of Owen *et al.* (1940) showed that these different types of elderly people could be as readily distinguished by their Ca balances as by their P balances (Figs. 1 and 2). This is only to be expected if the real cause of variation from person to person is the initial degree of depletion of the skeleton. In a person with a fully mineralized skeleton, over four-fifths of his P and 99% of his Ca are in his bones (Shohl, 1939). Fairbanks & Mitchell (1936) suggest that subjects under test should have their Ca and P stores filled by dietary treatment before determining their Ca and P requirements. This is important in view of findings by Orr & Clark (1930) that the greater the monetary income the greater is the Ca intake.

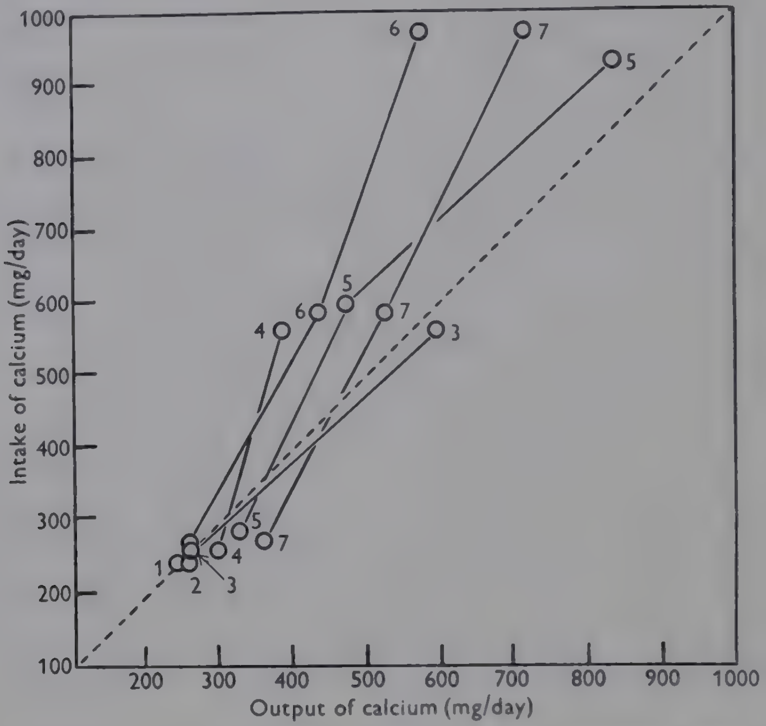

Fig. 1. Calcium balances of elderly men (from Owen, Irving & Lyall, 1940).

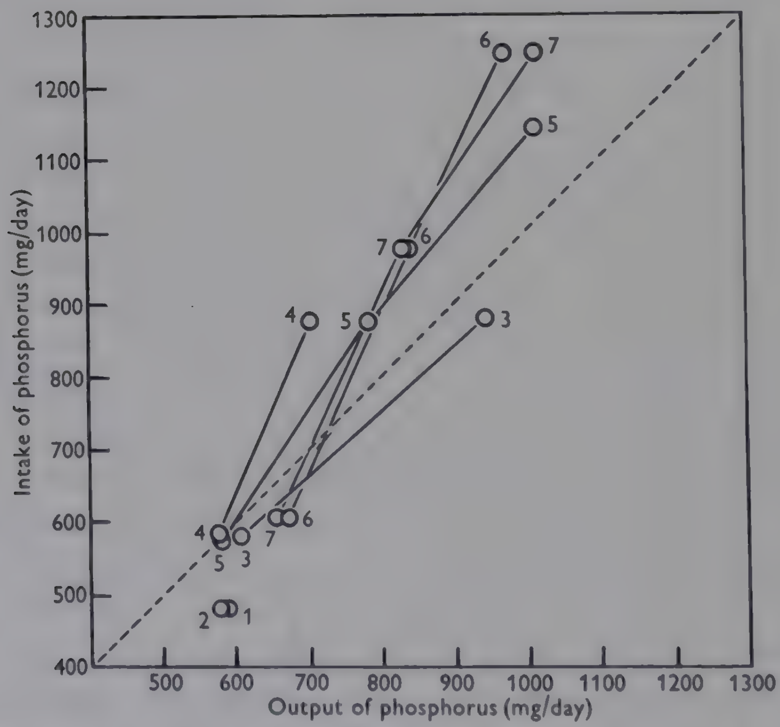

Fig. 2. Phosphorus balances of elderly men (from Owen, Irving & Lyall, 1940).

A certain optimum content of fat in the diet is favourable to the storage of Ca and P in bones, even on a diet already adequately supplied with vitamin D. Loss of Ca through the bowel is accompanied by excessive loss of Ca soaps at the same time

(Steudel, 1944). Ca is lost through the bowel in coeliac disease, in idiopathic steatorrhoea (Hunter, 1930*a–c*) and in sprue. Such Ca loss also occurs if fat of too high a melting-point is used in the manufacture of margarine (Chanda, 1949; Westerlund, 1947; Dutta, 1948). Butter (Steggerda & Mitchell, 1951) and margarine of suitable melting-point do not have this effect.

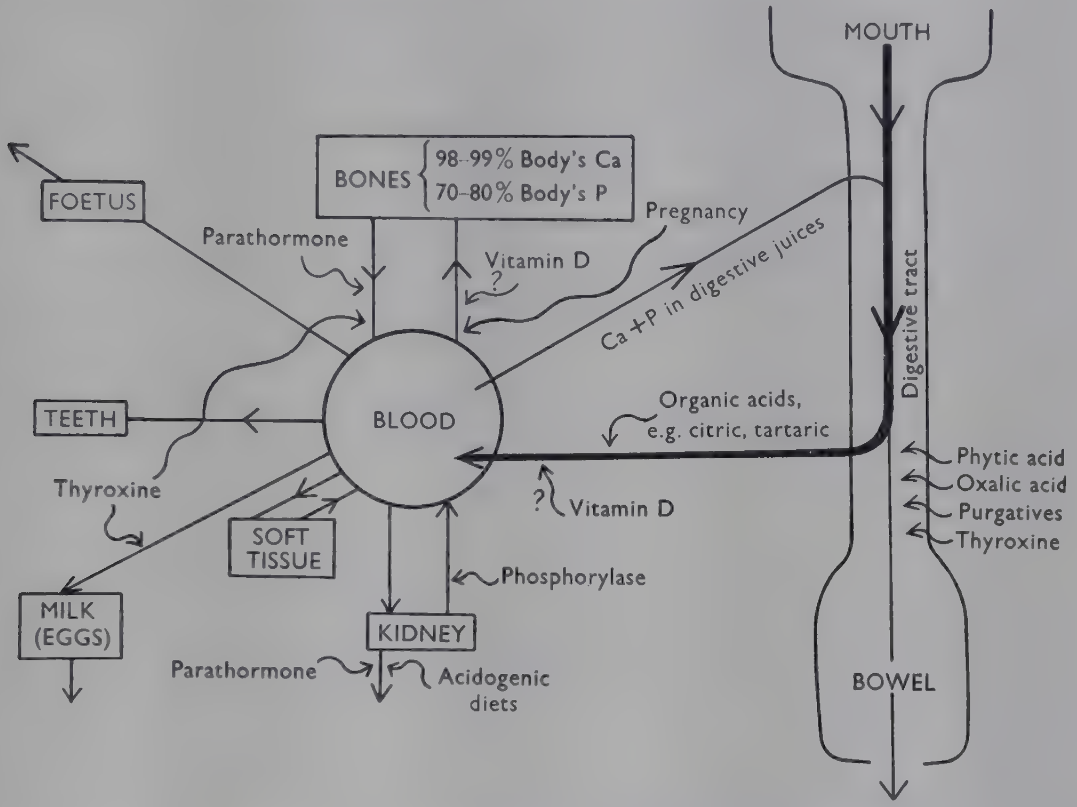
MOUTH
FOETUS
BONES
98–99% Body's Ca
70–80% Body's P
Pregnancy
Parathormone
Vitamin D
?
Ca+P in digestive juices
Digestive tract
TEETH
BLOOD
Organic acids,
e.g. citric, tartaric
Phytic acid
Oxalic acid
Purgatives
Thyroxine
Thyroxine
?
Vitamin D
SOFT TISSUE
Phosphorylase
MILK (EGGS)
KIDNEY
Parathormone
Acidogenic diets
BOWEL

Fig. 3. Diagrammatic representation of metabolism of calcium and phosphorus. The wavy arrows indicate acceleration. No attempt has been made to segregate action upon Ca from action upon P. Since the composition of bone makes it difficult for either of these elements to be laid down in bone or be withdrawn from bone without the other, the composition of bone salts is generally regarded as constant (Follis, 1952) unless metabolism of bone is seriously deranged (Stewart, 1932–3, 1934–5). The diagram is consistent with facts cited by Best & Taylor (1950) but the interpretations are somewhat different. The present author believes that the maintenance of blood Ca to prevent tetany takes priority over adequate skeletal mineralization so that rickets results if the intake of Ca and P is too small.

Ewer & Bartrum (1948) and Grant (1951) have studied the production of rickets that occurs when young animals are allowed to graze young green oats. It is possible that such young green material contains over 30% protein (Owen, 1943) in a very digestible form and that its power to produce rickets is thus either generated or aggravated by an excessive stimulation of growth by a diet rich in protein, rich in digestible carbohydrate, poor in calcium and entirely lacking in vitamin D. If this theory is correct such rickets should be of the low-Ca type and should be aggravated by supplementation with P or prevented by administration of calcium lactate. Grazing animals may be subjected to wide variations in the Ca:P ratio of their diets. This ratio will be small in spring-time when the grass is young and large in autumn when it is old, for, as grass ages, it becomes richer in Ca and poorer in P (Waite, Holmes, Campbell & Fergusson, 1950). Feeding of seeds (oats and beans) undoes the effect of this disparity by providing P partly as phytate which is, however, a good source of P for the ruminant because phytate is digested by the rumen bacteria (Reid, Franklin & Hallsworth, 1947). Seeds have a ratio of P to Ca which offsets the large Ca:P ratio of roughage.

SUMMARY

In conclusion, it may be said that the body's stores of calcium and phosphorus in the bones are labile and can only be filled in the young growing organism if the Ca and P in the diet bear a favourable ratio to one another (see Fig. 3). In animals on diets otherwise adequate, but deficient in Ca, P or Mg the soft tissues compete with the bones for Ca and P or Mg so that mineral is lost from the skeleton in adults. In the younger organism an undermineralized skeleton, which may be rickety, is formed. Vitamin D protects the organism by favouring the bones at the expense of the soft tissues. In early lactation the mammary gland draws heavily on the stores of Ca and P in the skeleton. The mammary gland thus resembles the other soft tissues, including the foetus and the developing egg. The Ca, P and Mg of the teeth seem to be, at least in part, protected from undue competition with the body's soft tissues. Mineral in the teeth is irreversibly deposited.

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Bone and Toxic Minerals

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During the years between the wars, we have seen the development of the spectrograph and of sensitive colorimetric methods for the identification and determination of elements in biological materials. As a result it has been established that animal and human tissues contain elements whose existence in the tissues had not previously been recognized. The concentration of these elements is low in comparison with that of such elements as sodium and potassium. It is quite possible that, if more sensitive methods could be devised, other elements would be recognized in even more minute concentrations.

It is of some interest to consider the reasons stimulating such work. In certain instances it was simply a desire to determine the maximum number of elements, even in low concentration, in animal and human tissues. At the same time our knowledge of the nutritional requirements of the animal body was being extended. During this period it was established that copper was a constant constituent of animal tissues and, although present in low concentration, was an essential element. Increased interest was also being taken in industrial toxicology. It became necessary to develop more sensitive and reliable methods for the identification and determination of toxic elements in excreta and in tissues.

Certain of these elements occur constantly, whereas others are found only spasmodically. Elements such as copper and zinc have been shown to be essential for normal animal and human metabolism. Lead, arsenic and fluorine, elements known for their toxic properties, are present in normal animal and human tissues. There are elements now known to be present in tissues in low concentration, which, in the present state of our knowledge, are without function. These elements are derived almost entirely from foodstuffs.

Certain toxic elements are deposited in the skeleton in preference to the soft tissues. Of these, fluorine, lead and radioactive metals of high molecular weight have received most attention.

Radioactive metals

The continued absorption of radioactive metals of high molecular weight has a cumulative effect, resulting in increasing concentrations in the skeleton. The effect of the radiations is disastrous. The continual bombardment of the bone marrow results in a profound and leukopenic anaemia. In addition, both the upper and lower jaws develop destructive lesions similar to those seen in phosphorus poisoning and due, as in that disease, to bacterial infection from the mouth superimposed on a specific osteitis. There has been comparatively little metabolic work carried out in this condition. Work in this field has been mainly of a preventive nature.

Fluorine

Fluorides occur in small amounts in the normal diet and in drinking water. Small quantities occur in normal tissues, the highest concentrations being found in the skeleton. There is no certain evidence that the small quantities of fluoride in normal tissues exert any essential function. There is some evidence, however, that, when optimal amounts of fluoride are available during growth, there is increased resistance to dental caries.

A moderate excess of fluoride results in dental fluorosis—an unsightly mottling of the teeth. Great excess of fluoride, which can be derived from drinking water, results in gross skeletal changes with severe disabilities and sometimes death. Such pathological changes are permanent. It has been demonstrated that the fluoride content of bone increases with age (Murray & Wilson, 1946). Comparatively little metabolic work has been carried out on subjects in this condition. Work in this field has been mainly of a preventive nature.

Lead

The factors influencing the absorption and distribution of lead in the animal body have received relatively more attention. There are several reasons to account for this. Lead poisoning has been on a very much wider scale, especially in industry. Lead poisoning is not usually a fatal condition and, with successful clinical treatment, the body is not subject to permanent pathological change. Since lead poisoning may respond successfully to treatment, the condition has received much attention not only from the biochemist, but from the clinician. We are indebted to Aub, Minot, Fairhall & Reznikoff (1926) for much of the earlier work on the metabolism of lead.

Lead is present in measurable quantities in 'normal' tissues (Table 1). These are derived almost entirely from the diet, which supplies daily about 0·5 mg. Lead tends to be deposited preferentially in the skeleton. Bones such as femur and tibia, however, tend to contain higher concentrations than rib or vertebra. It has been observed that the concentration of lead in femur and tibia increases with age (Lynch, Slater & Osler, 1934; Tompsett & Anderson, 1935; Tompsett, 1936). The concentration of lead in the soft tissues and such bones as rib and vertebra is unaffected by age. Such findings suggest that, under so-called 'normal' conditions, there is a retention of lead in certain parts of the skeleton.

Table 1. *Lead content of 'normal' human tissues*

(Tompsett & Anderson, 1935; Tompsett, 1936). The figures are expressed in mg Pb/kg fresh tissue)

Soft tissues		Bones	
Liver	0·9–4·6	Rib	5·0–12·9
Kidney	0·7–3·7	Vertebra	2·6–14·7
Spleen	0·4–5·9	Femur	18·2–108
Brain	0·2–0·7	Tibia	15·3–96·5

Table 2. *Lead content of tissues of persons with a history of abnormal exposure to lead*

(The results are expressed in mg Pb/kg fresh tissue)

Occupation ...	Painter	Painter	Metal worker	(Condition due to contamination of drinking water with lead)
Age (years)	41	60	60	26
Liver	4·5	2·4	5·4	7·1
Kidney	1·0	2·9	2·0	4·6
Brain	1·0	—	1·4	3·1
Rib	119	22	51	52
Vertebra	19	9	13	—
Femur	—	—	51	52
Tibia	—	—	79	53

I have had the opportunity to examine tissues taken *post mortem* from four persons with histories of abnormal exposure to lead (Table 2). The markedly increased concentration of lead in the ribs is particularly noteworthy.

Factors influencing the distribution of lead between the soft tissues and the skeleton are of particular interest to the clinician. It is generally assumed that only lead present in the soft tissues exerts a toxic action. The 'inert' lead of the skeleton is, however, a potential danger if mobilized and passed to the soft tissues. Mobilization of lead may be assessed either by an increase in its excretion or by an increase in the level in the blood.

It would appear that the so-called 'normal' lead of the skeleton is difficult to mobilize by metabolic means. On the other hand, lead recently deposited in the skeleton as the result of abnormal exposure is relatively easily mobilized by metabolic means. Such mobilization may result in the onset of toxic symptoms. In many respects lead resembles calcium. A high-calcium diet favours deposition of lead in the skeleton, whereas a low-calcium diet, with or without parathyroid hormone therapy, has a reverse effect. Acidosis, which can be produced clinically by the administration of ammonium chloride, also favours the release of lead from the skeleton.

Recently absorbed lead is said to be stored in the bony trabeculae and, in time, it is deposited in the cortex of the bones as the less soluble tertiary phosphate (Gant, 1938*a*, *b*). Such could account for the two different ways in which lead appears to exist in the skeleton.

The question naturally arises as to whether the lead of the skeleton, particularly that laid down under 'normal' conditions, can be mobilized as the result of disease. This applies particularly to those diseases having a gross effect on the skeleton. Brown (1946) and Tompsett (1948) have shown that mobilization of lead, as indicated by an increase in the lead in the blood, can occur in the following conditions: (1) involvement of the skeleton by malignant disease; (2) senile osteoporosis; (3) marrow hyperplasia—pernicious anaemia, leukaemia. In such persons, with no history of abnormal exposure to lead, there were no definite clinical symptoms of lead poisoning. In one group, the urinary coproporphyrin levels were determined. These levels are usually elevated in lead poisoning, but were found to be within normal limits in these special cases. The probability is that, in most cases, the release of lead from the skeleton, as the result of the above disease processes, is comparatively slow. In persons with a history of abnormal exposure to lead, the result of such diseases may however be more serious. A case of subacute lymphatic leukaemia, with a history of abnormal exposure to lead, has been reported in which symptoms of lead poisoning appear to have been precipitated by the disease (Brown & Tompsett, 1945).

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Radiology of Bones in Health and Disease

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Text not received for publication.

SEVENTY-THIRD SCIENTIFIC MEETING
DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF MANCHESTER

22 MARCH 1952

DIET IN RELATION TO RENAL AND CARDIO-VASCULAR DISEASE

Chairman: Dr JOHN WILKINSON, *Department of Haematology, Manchester Royal Infirmary*

Introduction to the Subject of Diet in Hypertension and Renal Disease

By R. Platt, *Department of Medicine, University of Manchester*

Dr Roscoe (1952) deals fairly comprehensively, as far as one can in a short space, with questions of diet in chronic renal disease. As to diet in hypertension, I feel there is very little to say. The effect of salt depletion (with which Black (1952) deals) is interesting but, as he says, it is doubtful if it has much practical importance in treatment. The only other dietetic measure, which is sometimes important, is to reduce the weight of hypertensive patients if they are fat. Sometimes this brings down the blood pressure, but even when it does we are not certain that this is a true effect because it is known from intra-arterial readings of blood pressure that measurements taken with the ordinary cuff round an obese arm are very inaccurate, and the effect of the diet may simply be that we are now obtaining blood-pressure readings more consistent with the facts. In any case the reduction of weight does good by relieving the heart of some of its work.

There are two main determinants of water excretion under normal circumstances. The first is the solute load, that is the total amount of dissolved substances which the kidney is called upon to excrete. McCance (1945–6) showed, and Rapoport, Brodsky, West & Mackler (1949) confirmed, that when osmotically active substances such as salt, urea or sucrose are given to a person deprived of water, the amount of urine increases very greatly. As it increases, its concentration, or osmolarity, becomes less, approaching the osmolarity of blood plasma. This is the condition known as osmotic diuresis. The second factor determining water excretion comes into play when water is present in the body in excess. This is what we know as water diuresis, and in this case the concentration of the urine approaches that of distilled water.

In renal failure—I am referring to patients in the late stages of renal disease without oedema—the excretion of solutes continues to be efficient in spite of the fact that perhaps only 10 or 20% of the renal tissue is still functioning. Thus the solute load per nephron or per unit weight of renal tissue is greatly increased and the kidney behaves as if it were continually under the stress of an osmotic diuresis (Platt, 1951). A concentrated urine therefore cannot be produced and, as is well known, the urinary specific gravity approaches that of the plasma less its proteins (about 1009).

On the other hand, water diuresis is inefficient and the kidney has difficulty in getting rid of a big water load such as that of a litre of water.

Thus, in determining the amount of fluid that we should give to these patients we have to bear in mind that the kidney needs water in considerable quantity in order to excrete its solutes with the minimum of energy expenditure. About 3 l./day is probably the optimum quantity. On the other hand, excess of water should be avoided. Fortunately the thirst mechanism guides the patient effectively in most cases, and so long as he is provided with fluid in sufficient quantity he is usually quite capable of judging for himself the amount that he should drink.

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Salt and Hypertension

By D. A. K. BLACK, *Department of Medicine, Royal Infirmary, Manchester*

Both the substantives of my title stand in some need of definition. ‘Salt’ is used not as a chemical expression, but as the ordinary description of sodium chloride; there is fairly general agreement that sodium is more important than chloride in sustaining blood pressure, and this is not without practical importance, as some of the ‘salt substitutes’ on the market, though free from chloride, contain sodium citrate. ‘Hypertension’ denotes a physical finding, that of raised blood pressure, and not a disease state; as clinical knowledge advances, we are able to distinguish patients in whom renal disease, suprarenal disease, or disease of the central nervous system accounts for the elevated blood pressure. Most patients with raised blood pressure have, however, no demonstrable primary disease to account for their hypertension; they are said to suffer from ‘essential hypertension’, and it is with this limited group of hypertensive patients that I propose to deal. Sustained hypertension can in itself cause damage to the kidneys, heart, and brain; most of the patients from whom we have derived data have a normal excretory function, but in one this was reduced to about a quarter of normal.

The practice of salt restriction in hypertension antedates considerably any acceptable rationale for the procedure. Widal and his school seem to have introduced it about 50 years ago, but its use in the English-speaking world dates from the enthusiastic advocacy of Allen in America in the nineteen-twenties. Hypertension is a syndrome in which the actual blood-pressure level is very variable, and there may be a considerable fall in blood pressure when the patient is removed from active life, especially if he is rested in bed. It is also a syndrome which is quite compatible with long survival after its initial discovery. Each of these considerations makes it difficult to assess the value of any proposed therapy, and until a few years ago the general opinion seemed to be that salt restriction of the type then practised could not be shown to influence the course or severity of the syndrome. From this statement, one must except those patients with hypertension of some standing and severity who are threatened with cardiac failure, associated with cardiac asthma or peripheral oedema; in them, salt restriction may alleviate the fluid retention found at this stage of the syndrome.

Interest has been revived by the work of Kempner (1944) on the rice and fruit-juice diet for hypertension. He claimed improvement in the blood-pressure level, and also in heart size, retinopathy, and electro-cardiographic findings. Attempts to confirm these findings in America met with varying success. In this country, a Medical Research Council trial (Anonymous, 1950) was made in a number of centres, and it was found that in about 70% of patients with hypertension a considerable fall of blood pressure could be produced. A diet of rice and fruit juices is, however, very monotonous, and though an obsessional patient may be induced to take it for long periods at the instance of an enthusiastic physician like Kempner, the usual hypertensive patient in this country soon gives it up. The rice diet represents severe restriction of sodium and chloride, with an intake of about 5 m-equiv./day. This is not without risk, especially if mercurial diuretics are also used, and Schroeder (1949) has reported a number of patients who became seriously depleted of salt, with low plasma volume and poor renal function in consequence.

The rice diet is probably impracticable for long-continued use. On the other hand, the usual 'salt-free' diet supplies about 3 g salt daily, and salt restriction at this high level is probably without effect on the blood pressure. By using specially prepared salt-free bread, a diet with less than 1 g salt daily can be provided without undue monotony, and this may possibly find some place in the treatment of hypertension. At present, interest has shifted to the methonium group of drugs, which are probably capable of producing a larger chronic fall in blood pressure than can be accomplished by salt restriction.

Although the rice diet is scarcely a practical therapeutic measure, the trials carried out in this centre gave us the chance of comparing the response to it in normal and hypertensive subjects. In neither group was there any unequivocal change in plasma-sodium levels; and although we have not studied this extensively, there does not seem to be any fall in plasma volume. We were specially interested in comparing the renal conservation of sodium in normal and hypertensive subjects, in view of the claims made by Perera & Blood (1946) that hypertensive patients conserved body fluid more effectively than normal controls, when subjected to salt restriction. Their studies covered only a 24 h period of salt restriction, and they based their conclusion mainly on change in body-weight and urine output. In only two subjects was the clearance of sodium and chloride measured, and it is said to have been reduced in the hypertensive patients; this would imply enhanced conservation of sodium.

Our own studies were made on two normal and five hypertensive subjects; one of the normals had three separate experiences of the rice-fruit diet. Specimens of urine were collected every 24 h on the rice diet, and the sodium output on successive days was determined. The sodium excretion in both groups fell to levels of 5–10 m-equiv./day, and the groups did not differ significantly in the level reached. Increase in urine volume, which Perera & Blood found in their normal group, but not in the hypertensive group, occurred in our series in two experiments with normal and three with hypertensive subjects. Our main conclusions, however, are based on the rate of decline of sodium output in the urine over the first 5 days of salt restriction. If Q_t is the 24 h sodium output on day t, and Q_0 the initial sodium output, then

$$Q_t = Q_0 \times \epsilon^{-\alpha t},$$

α being a constant which expresses the rate of disappearance of sodium from the urine. The value of α is given by the slope of the regression line of the quantity $-\log_\epsilon Q_t/Q_0$ on time. We have calculated this regression for our normal and hypertensive people and the numerical values are as follows:

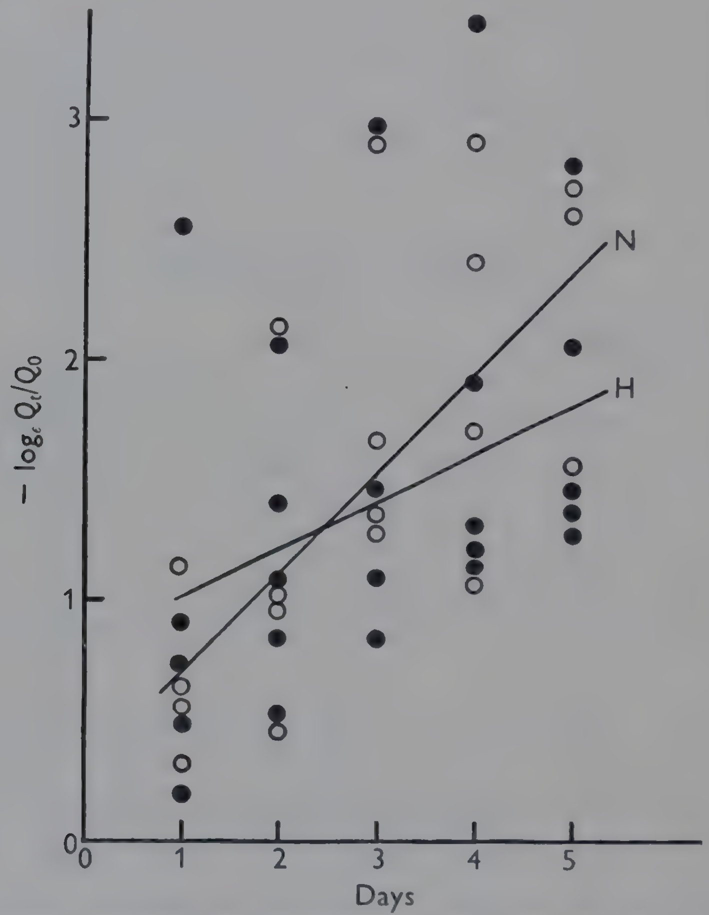

Fig. 1. Plot of the quantity $-\log_\epsilon Q_t/Q_0$ against time. ○, data from normal people; ●, data from hypertensive patients.

Experiments with normal people

$$\hat{Y} = 0{\cdot}19 + 0{\cdot}44X,$$

where $Y = -\log_\epsilon Q_t/Q_0$ and $X = t$.

The standard error, S_b, of the regressive coefficient $b = 0{\cdot}44$ is 0·098, so that the limiting values for $P = 0{\cdot}05$ are 0·24–0·64. (See also Fig. 1.)

Experiments with hypertensive patients

$$\hat{Y} = 0{\cdot}81 + 0{\cdot}22X,$$

$$S_b = 0{\cdot}11.$$

Limits for $P = 0{\cdot}05$ are 0–0·45. (See also Fig. 1.)

These results would seem to indicate that hypertensive subjects do not conserve sodium more effectively than normal subjects, and it is possible that they do so less effectively. Our results are thus in conflict with the conclusions of Perera & Blood (1946).

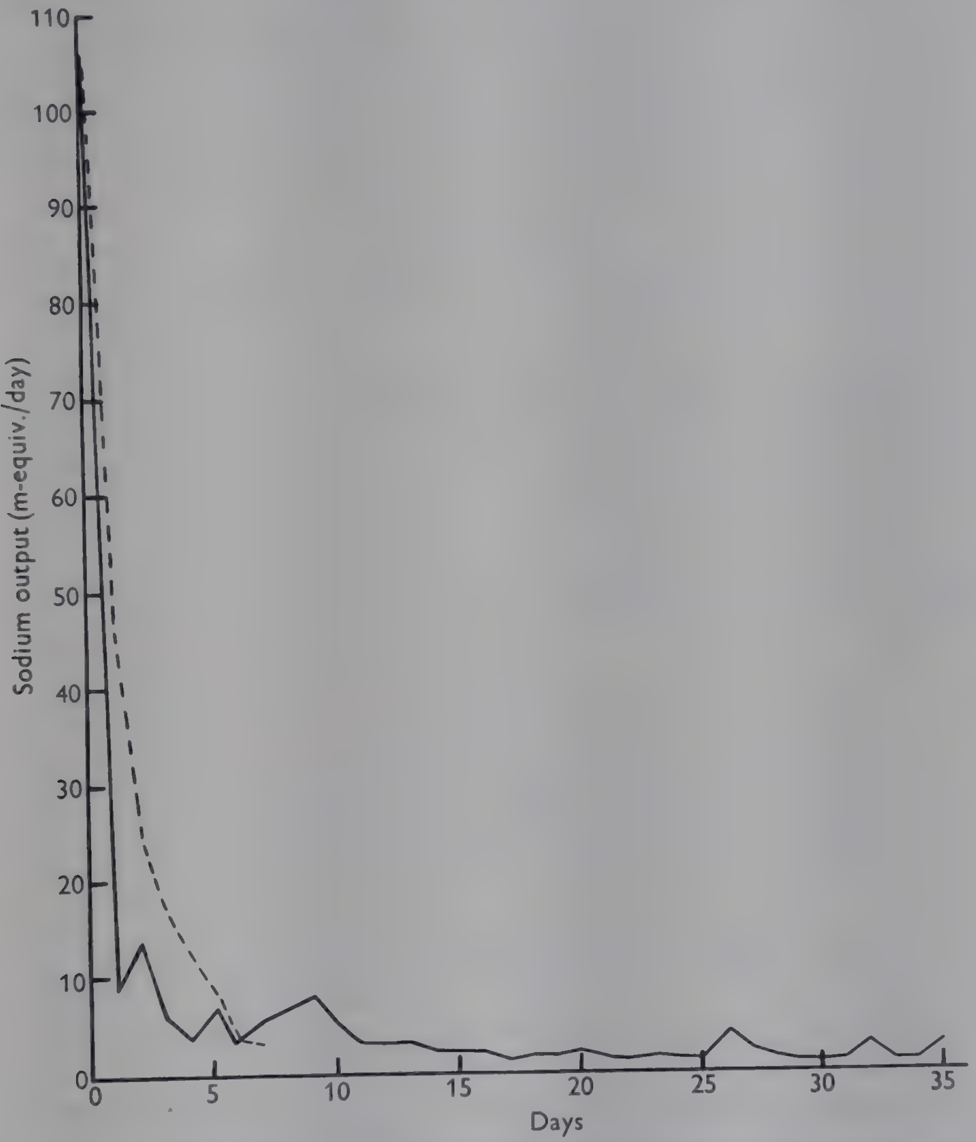

Fig. 2. Comparison of the decline in daily sodium output on the rice diet in a normal (---) and an exceptional hypertensive subject (——).

We should point out, however, that one of our hypertensive patients showed a remarkable power of salt conservation, as shown in Fig. 2, but even including her in the series, as we have done, does not bring the general hypertensive performance up to normal levels. The one patient with depressed renal function did not fail to conserve salt.

The method of computation adopted compares each day's excretion with the initial day's output, and so might be biased if there were an undue collection error on the 1st day of salt depletion. As a check on this, we have also taken the ratio of sodium output on the later days (Q_n) to the sodium output on each preceding day (Q_{n-1}). The average $Q_n:Q_{n-1}$ for normal people is 0·64, and for hypertensive patients 0·82, which is in accord with the more detailed results presented.

It has been claimed that overproduction of salt-retaining adrenal steroids is concerned in the production of essential hypertension, and Perera & Blood (1946) relate the failure of hypertensives to lose salt and water on a salt-free diet to this. In experimental hypertension (Floyer, 1951) the effect of adrenalectomy in bringing down the blood pressure can be prevented by supplying salt. It would seem from this and from previous work that the ability to conserve salt is essential to maintainance of blood pressure; but our present results do not support the view that the 'cause' of essential hypertension is an overproduction of the hormones which mediate salt conservation.

SUMMARY

1. The practice of salt restriction in the treatment of essential hypertension is discussed.

2. Five hypertensive patients did not conserve sodium more efficiently than normal subjects, when placed on a low-salt diet.

3. This study does not support the view that an excess of salt-retaining steroids is present in essential hypertension.

I am indebted to Professor R. Platt for the results on sodium output on the rice diet.

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Diet and Chronic Renal Disease

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Chronic renal disease includes a number of conditions that must be diagnosed before treatment can be carried out, but here only two will be considered; chronic type 2 nephritis and the chronic renal failure which develops following acute type 1 nephritis, chronic pyelonephritis or malignant hypertension.

Chronic type 2 nephritis

This is the only variety of chronic renal disease in which recovery occurs, so that treatment is particularly worth while. But although dietetic treatment would clearly appear to be indicated and may improve the symptoms, we can seldom be certain that is has had any curative effect.

The condition is characterized by marked albuminuria, which results in lowering of the plasma proteins, especially albumin, and in protracted cases in normochromic anaemia (Roscoe, 1950). There is also oedema, often of a very severe degree and, with this, sodium retention.

Treatment consists of a high protein intake to improve the plasma-protein concentration, and a low salt intake in the hope of reducing the oedema. The water intake does not seem to be important, and there is one form of treatment, which consists of giving 7 pt. of fluid a day, which sometimes results in diuresis with loss of the oedema.

Patients that do not recover sometimes lose their oedema and pass into a stage of chronic renal failure. The dietetic treatment must then be changed.

Chronic type 1 nephritis, chronic pyelonephritis and renal failure in malignant hypertension

The renal lesion in these conditions is always progressive, but the course may be prolonged, especially in chronic pyelonephritis, and the patients may lead a normal life for months, or even years, so it is important to prescribe any treatment that delays deterioration. There is degeneration of the kidney nephrons affecting both glomeruli and tubules and resulting in a quantitative loss of functioning kidney tissue (Platt, Roscoe & Smith, 1952) which becomes greater with time. At first the remaining nephrons are able to maintain excretion, but as more nephrons are lost kidney function fails.

It is not obvious why the degeneration of nephrons should continue when in many cases the original destructive process is no longer active, but a possible explanation is as follows: each remaining nephron has to carry out more than a normal amount of work in order to excrete an undue proportion of waste products, this leads to hypertrophy and thence to degeneration. This theory is supported by both pathological and experimental evidence.

Hypertrophy of the remaining nephrons in diseased kidneys is marked (Oliver, 1939) and it also occurs in animals subjected to partial nephrectomy (Addis, 1940) so that it is clearly a response to a reduction in kidney tissue. The extent of the hypertrophy, moreover, varies with the amount of work which the kidney remnants have to perform. This has been shown in two ways, both with partly nephrectomized rats. Firstly, the more kidney tissue is removed, the greater the work that each remaining nephron has to do, and Addis (1940) has shown that smaller kidney remnants hypertrophy more than larger ones. Secondly, the work of the kidneys can be increased by increasing the protein in the diet and so the amount of urea to be excreted, and Addis (1948) again showed that the hypertrophy was proportional to the protein in the diet. There is also experimental evidence that increasing the work not only produces hypertrophy but also hastens kidney failure. When a high-protein diet is given renal failure develops more rapidly and death occurs sooner both in partly nephrectomized rats (Addis, 1940) and in rats with nephrotoxic nephritis, an experimental condition resembling type 1 nephritis in its pathology (Farr & Smadel, 1939).

If, as the above results suggest, hypertrophy and degeneration are dependent on the work that the nephrons have to do, our treatment should be to reduce this work.

The actual energy expended in urine formation cannot be determined, but it is possible to calculate the mimimum amount of osmotic work needed for the excretion of each solute (Rhorer, 1905). This is:

$$W_{\min} = R \times T \times V(U \times \ln U/P + P - U),$$

where R and T = pressure and temperature constants, V = volume of urine/min,

U = concentration of the solute in urine and P = concentration of the solute in plasma. Now the work will become less the more nearly the urine approaches protein-free plasma in composition, and it is in fact found that in renal failure urine concentrations do not differ as widely from plasma concentrations as normally, which suggests that the available energy is limited.

When we consider the effect of different metabolites, it is found that urea and nitrogen and chlorine are together responsible for about 85% of the work and so are the only ones which need be taken into account. Urea is present in urine in much higher concentrations than in plasma, so that much energy is needed for its excretion. However, when the kidney tissue is reduced, at first all the urea formed cannot be excreted so the plasma concentration rises. This finally results in a normal rate of urea excretion, and it also means that the difference between plasma and urine concentrations is reduced and less energy is expended in excreting the same amount of urea, but even so the work involved is considerable. It is possible to reduce this work by decreasing the protein intake and so the urea excretion, and increasing the fluid intake so that the urinary urea concentration is reduced. Sodium and chlorine are usually consumed in such amounts that their urinary concentration is less than the plasma concentration, so here treatment should be by increasing their intake and reducing the fluid intake. Thus in the one case a high fluid intake is indicated and in the other a low. However, Newburgh (1943) has calculated that the best compromise between these conflicting needs is obtained when the daily fluid intake is about 2 l., and we now give our patients this amount of fluid and a low-protein diet with about 5 g of extra salt added to the food after cooking.

A few cases of chronic renal failure develop a condition characterized by marked loss of sodium chloride, and these must be given much larger amounts of salt (Thorn, Koepf & Clinton, 1944).

The need for a high salt intake must be remembered when renal failure develops in cases of hypertension treated with a low-salt diet. Here a salt deficiency may be more dangerous than a high blood pressure.

Anaemia of renal failure

In conclusion, the anaemia of renal failure should be mentioned. This increases in severity with increased nitrogen retention (Roscoe, 1952). It is not, like the anaemia of chronic type 2 nephritis, due to protein deficiency, so it is not adversely affected by a low-protein diet. It is normochromic in the majority of cases, so there is no point in giving iron. In fact, no effective treatment is known.

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Diet in Heart Disease

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Undernutrition is not an important factor in the causation of heart disease in this country, although elsewhere severe deficiency states such as beriberi may result in heart failure. A similar picture is seen in chronic alcoholics in whom there is a conditioned deficiency of vitamin B_1, but such cases seldom occur nowadays. Undernutrition may be a factor in the incidence of rheumatic fever, and a decline in incidence has been noted in countries like Sweden where standards of living have improved. It is probable, however, that better housing has been as important as improved nutrition in producing this reduction.

Diet has some part to play in the treatment of heart disease. It is important to remember, however, that a patient with heart trouble is of necessity limited in many ways, and it is unjustifiable to impose further restrictions unless there is good reason for them. Needless restriction, by producing introspection, is indeed often harmful. In a well-compensated ambulant patient there is no evidence that any particular kind of foodstuff is harmful, and we can advise as Sir James Mackenzie did, 'the kind of food the patient likes, so long as it does not disagree with him' (Mackenzie, 1908, p. 263).

When the heart fails, however, some modification of this attitude is necessary. Congestion of the liver and alimentary tract impairs the normal processes of digestion and absorption. The cardiac output is reduced, and the demands of the digestive tract on the limited amount of blood available must be curtailed. Finally, there is diminished excretion of sodium by the kidneys, with a tendency for the retention of salt and water in the tissues. In the presence of decompensation, therefore, one must advise small, frequent meals which can be easily digested and absorbed, with adequate rest after food. Restriction of salt is advised in order to avoid the retention of fluid in the tissues. A really salt-poor diet, containing less than 2 g daily, may be achieved in hospital, but it is unappetizing and difficult to prepare. At home, the most one can expect is for the patient to avoid salty foods, beer, and added salt at the table. This reduces the daily intake to approximately 5 g of salt and, when the salt intake has been previously high, may be sufficient to prevent the accumulation of oedema. In most cases, however, digitalis and mercurial diuretics are required as well.

Perhaps the most important contribution of dietetics to the treatment of heart disease is in the control of obesity, for the reduction of excess weight is a very valuable weapon in the treatment of all forms of heart disease, but particularly of coronary artery disease (angina pectoris and coronary thrombosis). Although obesity produces no actual structural damage to the heart, it may itself give rise to all the symptoms of heart disease, and when an organic lesion is present the additional burden of excess weight aggravates the symptoms and still further restricts activity. The basis of most reducing diets is a drastic reduction of fat and carbohydrate. The patient's co-operation is all-important and the ideal diet, therefore, should be easy to understand, not too difficult to adhere to and not too expensive. Reduction in weight is, at present, the single most effective measure we can employ in the treatment of coronary artery disease. It has been used on the assumption that a partly damaged heart will be more efficient if it has less weight to carry, but recent work raises the question whether dietary restriction of fat may not be of more fundamental importance.

Since the clinical syndrome of coronary thrombosis was first described (Herrick, 1912), it has steadily increased in frequency until it is now one of the most important causes of death in this country. This cannot be wholly explained by improved methods of diagnosis or by an ageing population. Any theory of aetiology must explain this increase, and must explain also why the disease is more common in men than women, in some races and countries than in others, and even in different occupations in the same country.

That coronary atheroma is an inevitable consequence of growing old is not borne out by the facts, and the influence of the 'stress and strain' of modern life is too vague a concept to be satisfactory. Atheroma is particularly common in conditions, such as diabetes and myxoedema, which are associated with a high blood cholesterol, and in familial hypercholesterolaemia, atheroma and sudden death from coronary thrombosis are common. Workers in America (Katz, 1952) have fed animals with a high-cholesterol diet and have succeeded in producing experimental atheroma. The proportion of cholesterol in these experimental diets, however, is far higher than is ever found in human diets, coronary atheroma is not always associated with a high blood cholesterol, and even when it is there is no evidence that the diet has contained an excessive amount of cholesterol. Despite the fact that coronary artery disease is frequently associated with obesity, there is little to suggest that an increased dietary intake of fat or cholesterol is important in producing atheroma. It is more probable that the metabolism of fat is at fault in some way, and recent work (Gofman, Jones, Lindgren, Lyon, Elliott & Strisower, 1950; Gofman, Graham, Lyon, Jones, Yankley, Simonton & White, 1951; Gofman, Jones, Lyon, Lindgren, Strisower, Colman & Herring, 1952) has shown that there is an increase in certain fractions of the serum lipoproteins in patients who have had a coronary thrombosis.

Further than this, one can only speculate, but environmental factors must play some part, and one is tempted to associate the increasing incidence of coronary artery disease with increasing mechanization and lack of physical activity. It is very probable that further information on the causation of coronary atheroma will be available shortly.

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Clinical Aspects of Nutritional Problems in Obliterative Vascular Disease

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Text not received for publication.

Physiological Aspects of Nutritional Problems in Obliterative Vascular Disease

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Text not received for publication.

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The Editors of the British Journal of Nutrition *accept no responsibility for the abstracts of papers read at ordinary scientific meetings of The Nutrition Society. These are published as received from the authors.*

ABSTRACTS OF COMMUNICATIONS

The Seventy-fourth Meeting of The Nutrition Society (Thirty-fourth of the Scottish Group) was held in the West Medical Theatre of the Royal Infirmary, Edinburgh 3, *on Saturday,* 19 *April* 1952, *when the following papers were read:*

Muscular Abnormalities of Newborn Calves in Relation to the Tocopherol Nutrition of their Dams. By K. L. Blaxter and F. Brown, *Hannah Dairy Research Institute, Kirkhill, Ayr*, and A. M. Macdonald, *Glasgow University and Royal Hospital for Sick Children, Yorkhill, Glasgow*

In an investigation of the causes of the seasonal and between-calf variations in the onset of muscular dystrophy under experimental conditions, the tocopherol nutrition, muscle chemistry and muscle histology of young calves, born in the late winter and early autumn have been studied.

The tocopherol content of the milk and of the blood serum of newly calved cows reflected the tocopherol intake of the cows before calving. The tocopherol level of the blood serum of 4-day-old calves was closely and directly related to the level in the blood serum of their dams. Placental transfer of tocopherols is considered to be of importance since appreciable amounts were found in the tissues of foetuses and newly born calves.

Chemical analysis of the urine and of the muscles showed that, in the late winter, some calves, irrespective of their tocopherol nutrition, had muscles low in creatine content and exhibited a pronounced creatinuria. The muscles of calves born in late winter contained less creatine than did the muscles of calves born in the early autumn. Many of the winter calves had muscles with hyaline foci of degeneration, and the lesions appeared to be comparable to the initial stages of dystrophic degeneration. The incidence was not closely related to the tocopherol nutrition of their dams. It is possible that the abnormality arises from interaction between tocopherol supply and some unknown environmental factor involving differences in nutrition, weather and management.

The Influence of Diet on the Development of the Alimentary Tract of the Calf. By K. L. Blaxter and M. K. Hutcheson, *Hannah Dairy Research Institute, Kirkhill, Ayr* and J. M. Robertson and A. L. Wilson, *West of Scotland Agricultural College, Auchincruive*

Quantitative information on the role of roughage in the development of the fore stomachs of calves was obtained with six Ayrshire bull calves purchased when 3–4 days old. One calf was killed immediately and the remainder were reared in two groups, with and without access to roughage, the allowance of whole milk being adjusted to ensure equality of skeletal and muscular development. Calves were embalmed in the natural standing position and dissected by stages from both left and right sides. Prior to death, there was a marked distension of the wall of the abdominal cavity in the group which received roughage, and on dissection it was apparent that roughage ingestion had considerably increased the capacity of the fore-stomach compartments. This had taken place without any increase in the weight of the walls, by 'stretching' of the tissues. Roughage ingestion, therefore, increased the capacity of those parts of the ruminant stomach which originate from an oesophageal dilation (see, however, Trautmann, 1931–2).

At 3 months of age the omasal volume of a calf given whole milk alone had increased five times, while one given roughage and milk had increased fifty-four times. The weights of both the small and large intestines, and the weight and capacity of the caecum and omasum, were unaffected by diet. The regional topography and juxtaposition of organs agreed with the findings of Lagerlof (1929) and Schmaltze (1895).

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The Response of Blood Amino-acids in the Fasting Subject to Glucose and to Fat Administration.

By H. N. MUNRO and W. S. T. THOMSON, *Department of Biochemistry, University of Glasgow*

The concentrations of amino nitrogen and of several of the essential amino-acids, were investigated in the blood of healthy adult human beings during fasting and following administration of glucose or fat. Blood samples were taken at four successive hourly intervals following a 15 h fast. When the subjects received no food during this period there was no significant change in amino-acid level of the blood. The giving of glucose caused a highly significant fall in the total amino-nitrogen level which was maximal at the 1st hour after administration. Following the giving of butter, the level fell very slightly.

The levels in the blood of the essential amino-acids we studied were all reduced after giving glucose, but the reductions were neither in equimolar quantities nor were they a percentage of the amount in circulation. Instead, the proportions in which the different amino-acids were affected showed a close similarity to the proportions of different essential amino-acids required by man (Rose, 1950). This suggests that the administration of glucose has an immediate influence on the rate of protein synthesis. Administration of fat does not have this action.

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The Effect on the Growth and Reproduction of Guinea-Pigs of Supplementing Diet 18 either with Cabbage or with Synthetic Ascorbic Acid.

By D. Brown, I. D. Ferguson and A. G. Ramsay (introduced by R. C. Garry), *Institute of Physiology, University of Glasgow*

One group of guinea-pigs was allowed an unlimited supply of cabbage outer leaves and of water, as a supplement to diet 18 (Bruce & Parkes, 1947). A corresponding group received 5 mg synthetic ascorbic acid orally, daily, in place of the cabbage. The ascorbic-acid content of the cabbage was estimated by dye titration (Johnson, 1948). The daily consumption of cabbage was noted. The guinea-pigs ate 50–90 g cabbage daily, providing an intake of 30–55 mg ascorbic acid. No significant difference in intake of cabbage was noted between males and females except during pregnancy, when the intake of the females was slightly increased.

Guinea-pigs allowed cabbage grew more rapidly and had a lower incidence of infection than the corresponding group receiving 5 mg synthetic ascorbic acid. Stillbirths and deaths during pregnancy were common in the females receiving 5 mg synthetic ascorbic acid, whereas females allowed cabbage did not die during pregnancy and produced larger and healthier litters. Females receiving 5 mg ascorbic acid had a calculated mean period from the probable first oestrus to fertile mating of 16 days compared with 2 days in the group allowed cabbage.

We wish to thank Roche Products Ltd. for the gift of ascorbic acid. The expenses were defrayed by grants from the D. C. Andrew Research Fund and the Rankin Medical Research Fund of the University of Glasgow.

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The Effect of Diet on the Average Protein and Nucleic-Acid Content of the Liver Cell.

By R. Y. Thomson, F. C. Heagy, W. C. Hutchison and J. N. Davidson, *Department of Biochemistry, University of Glasgow*

The effects of fasting, of a high-fat diet, and of diets deficient in protein and aneurin on the content of lipid phosphorus (LP), protein nitrogen (PN), ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) in the rat liver have been investigated. The average DNA content of the liver nuclei has been found not to undergo any statistically significant changes as the result of any of these treatments. The DNA content of the liver may therefore be taken as a measure of cell number and the ratios of the other constituents to DNA as a measure of average cell composition.

The following conclusions may then be drawn:

(1) The total number of cells in the liver was not significantly altered by any of the treatments used.

(2) Fasting for 48 h reduced the average cell mass by 40 % and the average cell

content of LP, RNA and PN by over 20 %. Feeding on an aneurin-deficient diet produced similar results.

(3) The protein-free and high-fat diets caused a 25 % fall in the average cell LP and RNA. With the protein-free diet (but not the high-fat diet) there was a corresponding fall in cell mass and PN.

The Partition of Carotenoids and of Vitamin A in the Colostrum of the Cow and of the Goat. By R. CHANDA, *Hannah Dairy Research Institute, Kirkhill, Ayr*

By chromatography, followed by spectrophotometry (Chanda, Owen & Cramond, 1951; Chanda & Owen, 1952), the partition of carotenoids and vitamin A in the colostrum of nine cows and six goats was determined.

Both the vitamin A and the β-carotene contents of the cow colostral fat decreased logarithmically with time during the first nine milkings. The decreases in β-carotene were more rapid than those in vitamin A. Cows at pasture secreted in their colostral fat more β-carotene than did cows on winter rations. The difference between the vitamin A content of the colostral fat of the two groups was not marked. Cows on winter rations had in their colostral fat a larger proportion of the total carotenoids in the inactive form than had the cows at pasture. Cow colostrum contained vitamin A alcohol during the 1st week of lactation.

Table 1. *The partition of carotenoids and vitamin A in the colostrum of the cow and the partition of vitamin A in the colostrum of the goat*

	Cow colostrum					Goat colostrum Vitamin A		
			Vitamin A					
No. of milkings *post partum*	Total carotenoids (μg/g fat)	β-Carotene (percentage of total carotenoids)	Ester (i.u./100 ml.)	Alcohol (i.u./100 ml.)	Total (i.u./g fat)	Ester (i.u./100 ml.)	Alcohol (i.u./100 ml.)	Total (i.u./g fat)
1	25·1	86	649	44	129	1252	67	195
2	20·9	79	544	40	96	944	53	172
3	20·4	75	489	30	91	902	48	157
4	22·7	63	412	36	84	783	0	145
5	16·5	65	345	22	70	720	0	123
6	12·8	78	294	17	65	530	0	112
7	9·8	81	252	15	58	479	0	95
8	8·2	76	214	12	49	329	0	75
9	6·4	78	182	12	41	316	0	69

The colostral fat of the goats on winter rations was richer in vitamin A than the colostral fat of the cows on winter rations. This superiority of the goat persisted when the lactation was established. Vitamin A content of goat colostral fat also decreased logarithmically with time. In the first three milkings, but not in later milkings, goat colostrum contained vitamin A alcohol. The mean results for three cows and four goats, all on winter rations, are summarized in Table 1.

β-Carotene was demonstrated in goat colostrum but by the eleventh milking it was no longer present.

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The Absorption of Certain Elements from the Alimentary Tract of Sheep.

By D. Parthasarathy (introduced by A. T. Phillipson), *Rowett Research Institute, Bucksburn, Aberdeenshire*

Absorption of volatile fatty acids from the rumen under experimental conditions is accompanied by movements of carbon dioxide and of chloride in the reverse direction. Otherwise little information is available regarding the permeability of the rumen epithelium to inorganic elements and other simple molecules. The concentrations of soluble potassium, sodium, chloride and inorganic phosphate ions in the rumen liquor in relation to the corresponding concentrations present in the blood leaving this organ suggest that sodium is absorbed against a concentration gradient; potassium, on the other hand, is absorbed on account of its high concentration in the rumen compared to its concentration in the blood. No evidence of net absorption of phosphorus was found, and, as far as can be judged from the blood picture, chloride appears to enter the rumen. In other regions of the alimentary tract potassium and sodium appear to be absorbed from the small intestine and caecum, while phosphate is absorbed from the omasum and small intestine and chloride from the small intestine and caecum.

The Existence in Human Urine of an Adrenocorticoid Specifically Related to Salt Metabolism.

By C. P. Stewart, J. S. Robson and S. L. Tompsett, *Department of Clinical Chemistry, University of Edinburgh*

One of us (S.L.T.) has devised a procedure which permits a 90 % recovery of deoxycorticosterone acetate (DOCA) added to urine whilst cortisone acetate does not interfere significantly. When the procedure is applied to normal urine the DOCA-like substances measured amount to about 5 mg per day (calculated as DOCA); the amount is smaller in cases of Simmond's and of Addison's disease, and is increased after administration of DOCA or ACTH but not after administration of cortisone.

There is a temporary increase in the excretion of the DOCA-like substances after surgical operation at the time when the excretion of potassium is increased. When patients, receiving a diet providing a constant daily intake of nitrogen, sodium, potassium and chlorine, are suddenly deprived of NaCl, there is shortly afterwards an increase, lasting for a few days, in the urinary potassium output and this appears to be paralleled by an increase in the excretion of the DOCA-like substances.

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ABSTRACTS OF COMMUNICATIONS

The Seventy-fifth Meeting of The Nutrition Society was held at the Linnean Society's Rooms, Burlington House, Piccadilly, London, W. 1, on Thursday, 15 May 1952, at 2 p.m., when the following papers were read:

Electrolyte–Water Relationships as a Means of Determining Changes in Body Water Content. By K. L. Blaxter and J. A. F. Rook, *Hannah Dairy Research Institute, Kirkhill, Ayr*

Storage of body fat can be determined from the equation: fat stored = body-weight gain − (protein + ash + water) stored. The lack of accurate methods for the estimation of water storage has limited this method to qualitative studies (Keys, Brožek, Henschel, Mickelsen & Taylor, 1950; McCance & Widdowson, 1951). A method for the accurate determination of water is now described. It is based on the assumption that the osmotic pressure of body fluids is constant and that sodium and potassium contribute a fixed portion of this osmotic pressure. Determinations of the sodium, potassium and water contents of muscle, fat, liver, erythrocytes, posterior chamber fluid and pericardial fluid of Ayrshire cattle, aged from 1 week to 4 years, gave the empirical relationship:

$$H_2O\ (\mathrm{g}) = 0{\cdot}2922\ \mathrm{Na}\ (\mathrm{mg}) + 0{\cdot}1471\ \mathrm{K}\ (\mathrm{mg}).$$

The standard error of estimate was ± 2·0%. No significant differences were observed between animals or between the above tissues. Brain and serum water were overestimated by 13% by this equation. The water of rumen, small intestine and caecum contents, of whole foetuses and of amniotic fluid was estimated accurately by the equation, but that of abomasal contents and of allantoic fluid was underestimated by 50%. Because of the discrepancies, water storage calculated from the above equation would, generally, be in error by about 0·5%, but during pregnancy an error of 12% could arise.

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The Indirect Estimation of the Water Balance of Animals as a Basis for the Determination of Energy Exchanges. By K. L. Blaxter and J. A. F. Rook, *Hannah Dairy Research Institute, Kirkhill, Ayr*

Prediction of water retention from body storages of sodium and potasium was studied in metabolism experiments with calves. Five experiments were conducted in a closed-circuit respiration chamber in which continuous determinations of oxygen consumption, carbon-dioxide and water-vapour production, mineral, nitrogen and energy balances were made throughout periods of 8 days. Water retention, estimated by the classical balance technique, was compared with that estimated from simultaneous determinations of sodium and potassium retention (Blaxter & Rook, 1952). The results agreed within ± 3%. Fat retention determined from carbon and nitrogen balances was compared with that estimated from gains of body-weight, protein, water and ash. In these short-term experiments the indirect estimation of fat retention was complicated by inaccuracies encountered in weighing the animal, but in later experiments, in which a more satisfactory weighing technique was employed, the error was substantially reduced and was shown to decrease linearly with increasing length of experimental period. Provided that sufficiently long experimental periods are employed, these results suggest that metabolic balances of minerals and nitrogen may be used to estimate energy retention, and, if combined with determinations of the heats of combustion of foods and excreta, the total energy exchange.

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Vitamin C Reserves in Patients in a Mental Hospital. By Z. A. LEITNER,

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For the purpose of this study 173 patients (103 male and 70 female) were selected from a larger series. Saturation tests were carried out according to the procedure of Harris & Abbasy (1937) for varying periods up to 7 consecutive days using 2:6-dichlorophenolindophenol as a reagent. In the present series only 8% were saturated on the 2nd, 25% on the 3rd day; 67% were saturated on the 4th day or after, 40% on the 5th day or after, 16% on the 6th day or after and 7% on the 7th day or after. In confirmation of the findings of Harris & Olliver (1943) there was a definite seasonal difference. Thus, the majority of patients in the spring series became saturated on the 5th and 6th day, in the autumn series on the other hand between the 3rd and 5th day. The length of stay in hospital had a pronounced influence; about 70% of the patients in hospital for less than 2 years were saturated on the 2nd and 3rd day, whereas among patients admitted for 10 years or longer only 18% became saturated during this time. Whilst only about 8·5% of the patients admitted for less than 2 years failed to reach saturation until the 5th day, with patients in hospital for 10 years or more the corresponding figure was 58%.

On the basis of this preliminary study it is indicated that a large proportion of patients who had been for long periods in a mental hospital had a low vitamin C status.

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Histochemistry of Succinic Dehydrogenase.

By A. H. MALATY and G. H. BOURNE, *Department of Histology, London Hospital Medical College, London, E. 1*

Succinic dehydrogenase can be demonstrated histochemically by the method of Seligman & Rutenburg (1951). It depends upon the fact that if fresh frozen sections of tissues are incubated in succinate plus a suitable buffer and ditetrazolium chloride, the ditetrazolium acts as a hydrogen acceptor and becomes reduced to, and precipitated as, an insoluble blue diformazan pigment.

We have investigated this reaction in a number of tissues and have found that it was difficult to obtain any result with the tissues of animals which had been killed by anaesthesia. Good results were obtained from those of animals which had been killed by a blow on the head. Best results were obtained with kidney and skeletal and cardiac muscle, but liver gave poor results. Nervous tissue was intermediate.

The reaction was granular and the shape, size and distribution of the granules was identical with those of mitochondria.

In cardiac and skeletal muscle the reaction was localized in the sarcomes, which are derived from mitochondria. These results further support the conception of the mitochondrial localization of the enzymes responsible for aerobic metabolism of cells.

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The Home Preservation of Runner Beans Using Iodized and Other Salts.

By H. R. HINTON and DOROTHY F. HOLLINGSWORTH, *Scientific Adviser's Division, Ministry of Food, Great Westminster House, Horseferry Road, London, S.W. 1*

A preliminary experiment in 1950 showed that French beans preserved by the dry-salting method and stored for 2 months with free-running salt, iodized or non-iodized, were tougher, according to taste-panel judgement, and greener in colour than beans preserved with kitchen salt. The pH of the liquor from the beans preserved with the free-running salts was much higher than that from beans preserved with kitchen salt. Iodized salt produced the highest pH.

The experiment was repeated in 1950–1 with runner beans which were stored for 9 months. The earlier results were confirmed but this time the iodized free-running salt had a more marked effect on toughness and colour than the non-iodized salt. It also produced a liquor of slightly higher pH than non-iodized salt in either experiment and the iodized salt in the first.

The findings of the second experiment were confirmed in a more extensive experiment conducted in 1951–2, which showed in addition that the amount of pectin extractable from the beans varied inversely with the pH. This observation suggests that the calcium and magnesium salts present in free-running salts, and causing the increased pH, react on the pectin in the beans to form insoluble calcium and magnesium pectates. It is suggested that these are deposited in the middle lamella which is then not dissolved away on cooking.

There was complete destruction of ascorbic acid in all samples.

The Effect of Thiouracil and Thyroxine on the Composition of Milk.

By R. CHANDA, MARY L. MCNAUGHT and E. C. OWEN, *Hannah Dairy Research Institute, Kirkhill, Ayr*

Thiouracil caused a decrease in the lactose and an increase of the chloride in milk. Thyroxine had the opposite effect. An increase of chloride accompanied a decrease of lactose with advancing lactation in untreated cows. In both treated and untreated animals there was thus the expected close negative correlation between the lactose and the chloride so that the freezing point of the milk of all cows was constant, irrespective of treatment. Neither thiouracil nor thyroxine affected the creatine or creatinine in milk in spite of the marked increase, caused by thyroxine in urinary creatine (Owen, 1948). Creatine phosphate was not present in milk. Calcium in the milk was unaffected by either drug thus contrasting with phosphorus which was increased by thyroxine (Owen, 1948; Chanda & Owen, 1951) and decreased by thiouracil (Chanda & Owen, 1951). The expected effect of thyroxine in increasing milk yield was observed and fat content also increased. Thiouracil depressed the yield. Neither drug affected the sodium, potassium or magnesium in the milk.

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Vitamin A, Carotenoids and Tocopherol Levels in the Blood of Two Different Classes of Patient.

By Z. A. LEITNER, *Claybury Hospital, Woodford Bridge, Essex, and 52 Welbeck Street, London, W. 1*, and T. MOORE and I. M. SHARMAN, *Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*

Vitamin A was estimated by $SbCl_3$, carotenoids by yellowness and tocopherol by $FeCl_3$ in blood plasma collected for a year in London from healthy private patients, and in Claybury from Mental Hospital inmates. Table 1 shows that the mental patients averaged slightly lower than the private patients for vitamin A and tocopherol, and much lower for carotenoids. There were only minor seasonal variations. Male private patients, of mean age 46 years, had vitamin A higher and carotenoids lower than in females, aged 44 years. These sex differences confirm general experience. In the

Table 1. *Mean values for March 1951–February 1952*

	Private patients			Mental patients		
	No. of subjects	Range	Mean	No. of subjects	Range	Mean
Vitamin A (i.u./100 ml.)	219	51–297	148	159	12–364	118
Carotenoids (μg/100 ml.)	220	15–371	133	179	18–137	53
Tocopherol (mg/100 ml.)	64	0·60–2·91	1·31	175	0·56–3·25	1·15

nental patients, however, both vitamin A and carotenoids were slightly higher in vomen, aged 69 years, than in men, 49 years. Analysis of the data by age failed to xplain the unexpected vitamin A superiority in female mental patients. Possibly sex nly influences vitamin A levels at liberal carotenoid intakes. The carotenoid levels ow reported for private patients exceed previous British values, but are lower than n U.S.A. Vitamin A and tocopherol are in the same ranges as in U.S.A. Statistical alculations are proceeding.

A Suggested Modification of the 'Rat Growth' Method of Protein Evaluation.

By K. J. Carpenter and G. Porter, *Rowett Research Institute, Bucksburn, Aberdeenshire*

As the growing rat requires some protein for maintenance, the 'protein-efficiency ratios' of diets (body-weight gain ÷ total protein eaten) can be disturbed by differences in palatability with *ad lib.* feeding (see, for example, Mitchell, 1944; Graves, 1945). The variance of individuals is also high (Harte, Travers & Sarich, 1947). Paired-feeding techniques are unsuitable as the heavier animal has the greater maintenance requirement.

In an attempt to overcome these objections we have used: (1) A fixed range of growth (65–80 g), in contrast to other methods having a fixed period of growth. (2) A preliminary period of adaptation to the experimental diets (from weaning, at 45–52 g, up to 65 g, with *ad lib.* feeding to 60 g body-weight). (3) A scale of daily feeding according to body-weight (7·0 g/day for rats of 60 g, +0·3 g/day for every 2 g over this weight)—the highest possible for diets of medium palatability.

The variance between rats has been such that a 10% difference between two rations could be detected ($P > 0{\cdot}95$) with eight rats/treatment. As in all methods for the determination of 'protein-efficiency ratios' the values are relative to one another rather than absolute (cf. Hegsted & Worcester, 1947; Bureau of Biological Research, 1951), and will vary with the level of protein used in the diets (Barnes, Maack, Knights & Burr, 1945). They may also, because of variations in composition of the body-weight gained, differ in their ranking from 'net protein values' based on nitrogen retention (Mitchell, 1944).

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The Supplementary Protein Value of a By-product from Grass Processing.

By K. J. CARPENTER, J. DUCKWORTH and GABRIELLE M. ELLINGER, *Rowett Research Institute, Bucksburn, Aberdeenshire*

The moisture content of fresh grass intended for drying was reduced to 60–70% by an expelling operation. A by-product, Graminex, was obtained by heat coagulation from the expressed liquor (Dawson, 1951). It contained negligible crude fibre and 33–55% crude protein, with a high lysine and methionine content important in supplementary proteins (cf. Carpenter, 1951).

			Value in mixed rations					
			Chicks		Rats			
	Analytical data (g/16g N)							
	Lysine	Methionine	'Gross protein value'	Protein-efficiency ratio	Protein-efficiency ratio	True digestibility*	Biological value for growth*	Net protein value*
White fish meal	6·1	2·9	90	1·88	3·81	90	68	61
Groundnut meal	3·4	0·9	50	1·68	2·83	86	55	47
'Graminex'	5·2	1·8	56	1·22	2·35	80	63	50
Grass meal	4·9	1·6	55	†	†	†	†	†

* Mitchell, 1944. † Not available.

The 'gross protein value' (Heiman, Carver & Cook, 1939) of Graminex for chicks (3% supplementary protein) was superior to that of groundnut meal. With practical 19% protein rations (11% supplementary protein) it was inferior. The Graminex ration was then laxative, and protein and energy were less completely digested. With rats, using the same rations diluted to give 9·5% total protein, the protein-efficiency ratios followed the same trend. However, the 'net protein value' of the Graminex ration exceeded that with groundnut meal. In no case did Graminex approach fish meal in value.

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